APPENDIX 4

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Neuronal vulnerability in Parkinson's disease

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Summary. Although Parkinson's disease is characterized by a loss of dopaminergic neurons in the substantia nigra not all dopaminergic neurons degenerate in this disease. This suggests that some specific factors make subpopulations of dopaminergic neurons more susceptible to the disease. Here, we show that the most vulnerable neurons are particularly sensitive to oxidative stress and rise in intracellular calcium concentrations. Because both events seem to occur in Parkinson's disease this may explain why some dopaminergic neurons degenerate and other do not.

Which dopaminergic neurons degenerate in Parkinson's disease?

The main anatomobiochemical characteristic of Parkinson's disease is the loss of dopaminergic neurons in the mesencephalon. Degeneration of dopaminergic neurons is, however, heterogeneous in this structure, the degree of neuronal loss varying considerably from one catecholaminergic cell group to another. It is severe in the substantia nigra pars compacta (76% loss), intermediate in the substantia nigra pars lateralis (34% loss), ventral tegmental area (55% loss) and catecholaminergic cell group A8 (31% loss) but almost nil in the central gray substance (7% loss) (Hirsch et al., 1988).

Besides these inter-regional differences in the degree of neuronal loss in the mesencephalon, the loss of dopaminergic neurons has also been found to be variable within subgroups of neurons in the substantia nigra pars compacta. Indeed, in 1937, Hassler published a very precise description of the human substantia nigra, in which he subdivided it into more than thirty subgroups of neurons according to cytoarchitectonic and cell density criteria (Hassler, 1937). In Parkinson's disease, he found that the neuronal loss was the most prominent in the caudal and ventro-lateral part of the substantia nigra pars compacta (Hassler, 1938). More recently, Fearnley and Lees (1991) subdivided the structure into six main clusters of melanized dopaminergic neurons at a level of the substantia nigra pars compacta including the emerging fibers of the third cranial nerve. They found that the neuronal loss was massive in the

ventro-lateral part of the structure and less pronounced in its dorsal part. Nevertheless, the precise identification of the dopaminergic neurons that degenerate in Parkinson's disease requires further study for at least two reasons.

1. In most of the previous studies only melanized neurons were analyzed. Although the melanized neurons are dopaminergic in the substantia nigra (Bogert et al., 1983), they represent only a subpopulation of dopaminergic neurons, since 16% of all nigral dopaminergic neurons are devoid of neuromelanin (Hirsch et al., 1988).

2. The subdivision of nigral cell groups in the previous studies was essentially based on the dopaminergic neurons which degenerate during the pathological process. However, the use of limits which are dependent on the pathological process to identify the regions which degenerate in the diseased state is open to criticism. Indeed, the absence of neurons in the ventral portion of the substantia nigra may be due to a global atrophy of the substantia nigra or to a real and selective loss of the dopaminergic neurons in the ventral part of the structure. In order to circumvent these methodological drawbacks, we recently performed a precise analysis of the distribution of neuronal loss in the parkinsonian substantia nigra using calbindin immunoreactivity as a marker which is independent of the pathological process (Damier et al., 1996).

Although, calbindin immunoreactivity has been observed in a few nigral dopaminergic neurons mainly located in the dorsal part of the structure (Hirsch et al., 1992), most of the staining consisted of a dense fiber network covering the whole substantia nigra. This intensely stained neuropil was heterogeneous, showing pockets of lower staining intensity (Fig. 1). Interestingly, a great proportion of the dopaminergic neurons, identified by their neuromelanin content, were clustered together within the zone of calbindin-poor immunostaining. Five main calbindin-poor zones were identified in the human mesencephalon, which displayed a reproducible pattern of distribution across the different subjects analyzed. This heterogeneous pattern of calbindin neuropil was preserved in Parkinson's disease in agreement with the data of Ito et al. (1992). Using this definition of the dopaminergic neurons in the substantia nigra, which is thus independent of the pathological process, the neuronal loss was found to be more pronounced in the ventral substantia nigra (97% loss) than in its dorsal part (57% loss) (Damier et al., 1996). Moreover, the neuronal loss was also more severe in the calbindin-poor zones (95% loss) than in the surrounding calbindin-rich neuropil (80% loss). Furthermore, the neuronal loss was the most intense in the largest calbindin-poor zone (98% loss). This calbindin-poor zone was located in the ventral tier of the substantia nigra rostrally and was more lateral and dorsal, caudally.

Taken as a whole these data show that Parkinson's disease is not a generalized disease of dopaminergic neurons and that some doparninergic neurons are more susceptible to degeneration than others. Identification of the factors that render some dopaminergic neurons more vulnerable may thus provide new clues to the pathogenesis of the disease. Some of the phenotyp-

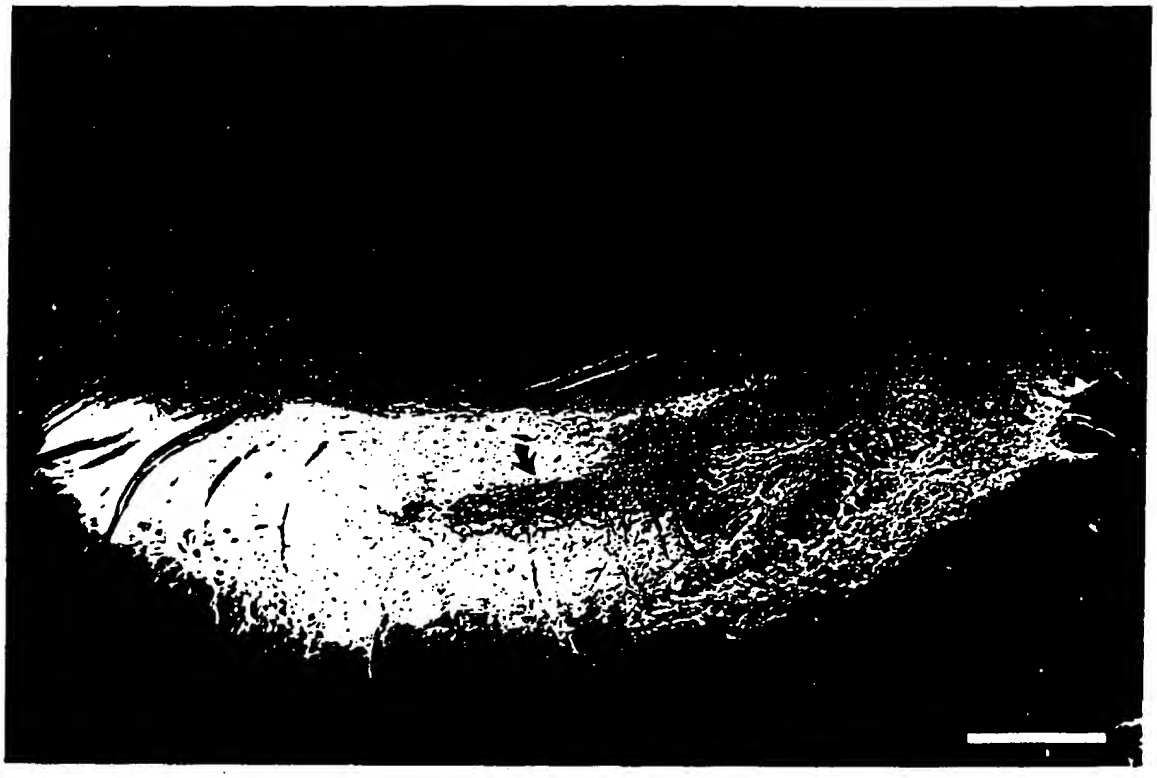

Fig. 1. Reverse contrast photograph of calbindin immunoreactivity in the mesencephalon of a control subject. Asterisk indicates a pocket of low calbindin immunoreactivity containing numerous melanized dopaminergic neurons (small white dots). Bar indicates 2 mm

ical differences between the dopaminergic neurons that are vulnerable in Parkinson's disease and those which are preserved are described in the next paragraph.

Vulnerability factors of dopaminergic neurons in Parkinson's disease

Various mechanisms may account for the selective vulnerability of some dopaminergic neurons in Parkinson's disease. Several lines of evidence suggest that the metabolism of oxygen free radicals and that of calcium is of particular interest. Indeed, increased intracellular concentrations of these two compounds have been shown to be involved in or to provoke nerve cell death under several different experimental conditions.

A higher production of oxygen free radicals and/or a low level protection against them in the regions most at risk may represent the best hypothesis to explain the role of oxidative stress in the selective vulnerability of some dopaminergic neurons. Indeed, two populations of dopaminergic neurons are observed in the human mesencephalon, those containing neuromelanin and

those without. This compound is synthesized by auto-oxidation of dopamine, a reaction which is associated with the production of highly toxic oxygen free radicals. The melanized dopaminergic neurons may thus produce more free radicals than those without melanin. Interestingly, the melanized dopaminergic neurons are found in the dopaminergic cell groups which are the most affected in Parkinson's disease, whereas the non-melanized ones are located in the least affected regions (Hirsch et al., 1988). This suggests that the dopaminergic neurons which produce the higher amount of oxygen free radicals are those which are the most vulnerable. Moreover, the most vulnerable dopaminergic neurons are also those which are poorly protected against oxidative stress. Indeed, all the mesencephalic dopaminergic neurons contain copper/zinc- and manganese-dependent superoxide dismutases, which are enzymes involved in the transformation of the superoxide ion into hydrogen peroxide in the cytoplasm and the mitochondria, respectively (Ceballos et al., 1990; Zhang et al., 1994). Yet, the density of glutathione peroxidase-positive cells is lower in the dopaminergic cell groups which are vulnerable in Parkinson's disease than in the cell groups which are preserved (Damier et al., 1993). Since hydrogen peroxide is mostly catalyzed by glutathione peroxidase in the brain and this compound is the main source of the extremely toxic hydroxyl ion, this suggests that the most vulnerable dopaminergic neurons in Parkinson's disease are particularly sensitivite to oxidative stress.

The dopaminergic neurons which degenerate in Parkinson's disease also seem to be particularly sensitive to a rise in intracellular calcium concentrations. Indeed, calbindin D28K, a protein that binds more than 95% of the intracellular calcium, has been found only in the dopaminergic cell groups that are preserved in Parkinson's disease and in non-human models of Parkinson's disease (Yamada et al., 1990; Manaye et al., 1991; Hirsch et al., 1992). The association between the presence of calbindin in dopaminergic neurons and their preservation in parkinsonian syndromes is even observed within the substantia nigra pars compacta, since the dorsally located neurons, which are more preserved, express this protein whereas the more ventrally located ones, which degenerate, are devoid of the protein. In addition, this relationship seems to be specific to calbindin, since it has not been observed for calretinin, another calcium binding protein (Mouatt-Prigent et al., 1994).

The data concerning the free radical defense mechanisms and regulation of the intracellular calcium homeostasis may explain why some catecholaminergic neurons degenerate in the mesencephalon of patients with Parkinson's disease and others do not. Such a hypothesis does not explain the primary cause of neuronal degeneration in the disease but may provide clues to identifying the cascade of events leading to nerve cell death. Indeed, it suggests that oxidative stress or increased intracellular calcium concentrations may be associated with nerve cell death. The data supporting this supposition are discussed in the next paragraph.

Factors possibly involved in nerve cell death in Parkinson's disease

The exact mechanism of nerve cell death in Parkinson's disease is still unknown. Among several hypotheses, it has been proposed that oxidative stress may participate in the nerve cell death. Indeed, it has long been known that oxygen reactive molecules can damage several biological molecules, including carbohydrates, proteins, nucleic acid and lipids (Fahn and Cohen, 1992). This is supported by the fact that an increased lipid peroxidation, interpreted as the consequence of an attack on polyunsaturated fatty acid by oxygen free radicals, has been reported in the substantia nigra of patients with Parkinson's disease (Dexter et al., 1989). Excessive amounts of oxygen free radicals can be produced when transition metals, especially iron, interact with hydrogen peroxide to form highly reactive hydroxyl ions. Interestingly, several studies have reported an increased iron content in the parkinsonian substantia nigra (Earle, 1968; Dexter et al., 1989; Jellinger et al., 1990, 1992; Hirsch et al., 1991; Good et al., 1992), suggesting a possible role of iron in the mechanism of nerve cell death in Parkinson's disease. This increase seems to be specific to (1) the altered dopaminergic cell groups, since it is not observed in the central gray substance which, is unaffected in Parkinson's disease, and (2) the disease, since it is not detected in the substantia nigra of patients with progressive supranuclear palsy, in which dopaminergic neurons also degenerate, probably through a different mechanism (Hirsch et al., 1991).This metal may be particularly active in inducing oxidative damage in the substantia nigra because, even in normal physiological circumstances, the neurons located in this structure produce large amounts of oxygen free radicals and are already poor metabolizer of free radicals (see above).

The origin of the increased iron concentration in the substantia nigra of patients with Parkinson's disease is not known. One of the possible causes could be a reduced concentration of iron-binding proteins, such as ferritin, or an increased cellular iron uptake. Ferritin levels have been measured in the substantia nigra of patients with Parkinson's disease, but contradictory results showing either reduced or unchanged ferritin levels in the substantia nigra of patients with Parkinson's disease have been reported (Riederer et al., 1989; Dexter et al., 1990). Such differences, which are probably due to the use of antibodies directed against the heavy or light chains of ferritin, call for more extensive studies of neuronal iron-binding capacities in Parkinson's disease. More recently, putative iron uptake mechanisms in this disease have also been analyzed. By analogy to the mechanism of iron penetration in the liver cells, a mechanism mediated by transferrin and its receptor might account for iron penetration into nigral dopaminergic neurons. However, this hypothesis is unlikely given the extremely low density of ^{125}I-transferrin binding sites in the human substantia nigra and their unchanged levels in Parkinson's disease (Faucheux et al., 1993). Alternatively, iron may gain access to dopaminergic neurons at the level of their terminals in the striatum and may be retrogradely transported to their cell bodies in the substantia nigra. Such a hypothesis is compatible with the high density of ^{125}I-transferrin binding sites observed in the striatum and their increased density in Parkinson's disease (Faucheux et

al., 1995). Caution is needed in interpreting these results, however, since the increased density of transferrin receptors may occur not only on dopaminergic terminals but also on those of other cell types. Lactoferrin (previously called lactotransferrin) and its receptor, which is specific for this type of transferrin, may also participate in iron penetration into dopaminergic neurons. Indeed, using specific antibodies we found the receptor for lactoferrin to be localized on neurons (pericarya, dendrites and axons), cerebral microvasculature, and, in some cases, glial cells in the human substantia nigra (Fig. 2) (Faucheux et al., 1995). In Parkinson's disease, the immunoreactivity was increased on neurons and microvessels in the substantia nigra and the other affected dopaminergic cell groups but not in the third cranial nerve nucleus, which is unaffected by the disease. Moreover, this increase was more pronounced in the most severely affected patients. These data suggest 1) that lactoferrin receptors may be involved in iron uptake in the human brain both at the level of the blood-brain barrier and on the cytoplasmic membrane of the neurons, 2) that alterations in this iron entry system may account for the increased iron levels in nigral neurons of patients with Parkinson's disease. A role of lactoferrin and its receptor in the succession of biochemical changes leading to nerve cell death is further supported by the fact that lactoferrin levels have also been found to be increased in the substantia nigra of patients with Parkinson's disease (Leveugle et al., 1996).

Besides oxidative stress, other factors are also thought to participate in the mechanism of nerve cell death. This is in particularly true of calcium, which can activate proteases or endonucleases and ultimately lead to cell death. As indicated above, the dopaminergic neurons which degenerate in Parkinson's

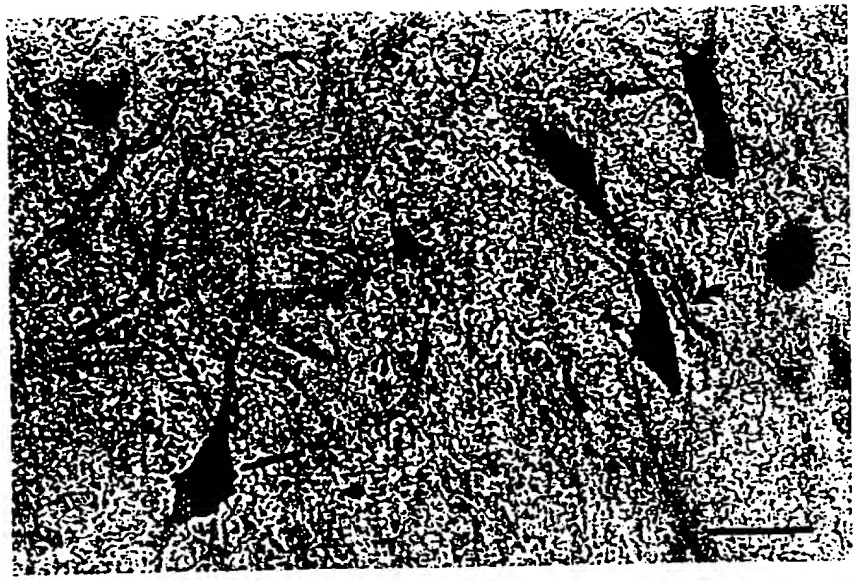

Fig. 2. Lactoferrin receptor immunoreactivity in the substantia nigra of a control subject. Note the presence of immunostained neurons (asterisk) and blood vessels (arrow). Bar indicates 50 μm

disease are particularly sensitive to a rise in intracellular concentrations. Yet, the involvement of calcium in the various events leading to nerve cell death in Parkinson's disease has yet to be proven. We addressed this problem by analysing the distribution and expression of the calcium-dependent protease m-calpain in the mesencephalon of patients with Parkinson's disease and that of control subjects. Two calpain types have been described, one referred to as calpain I or (μ-calpain), which is mainly concentrated in neuronal cell bodies (Sim et al., 1985) and is active at micromolar concentration, and the other, designated calpain II (or m-calpain), which is predominantly observed in axon tracts and glial cells and requires at least millimolar concentration of calcium (Nixon et al., 1986). Quantitative analysis of m-calpain immunoreactivity in parkinsonian patients revealed an increase in the number of m-calpain-positive neurons in the substantia nigra and locus ceruleus, compared to control subjects (Mouatt-Prigent et al., 1996). The density of m-calpain-positive fibers was also increased in both structures. Moreover, calpain-positive fibers presenting an abnormal morphology, and which were also ubiquitin-positive, were observed in the parkinsonian substantia nigra and locus ceruleus, suggesting that the presence of m-calpain is associated with degenerating neurons (Fig. 3). This is further supported by the presence of calpain immunoreactivity in the Lewy bodies, which are the histopathological stigmata of the disease. Furthermore, an unaltered density of m-calpain-positive neurons and nerve fibers was observed in progressive supranuclear palsy and striatonigral degen-

Fig. 3. Calpain immunostained nerve fibers displaying an abnormal morphology in the locus coeruleus of a patients with idiopathic Parkinson's disease. Bar indicates 100 μm

eration, suggesting that the increased calpain expression is specific to a given type of nerve cell death mechanism. However, this supposition must be treated with caution given 1) the loss of neurons expressing calpain in the cerebral cortex of patients with Alzheimer's disease (Iwamoto et al., 1991), 2) the presence of calpain in senile plaques and neurons bearing neurofibrillary tangles in the cerebral cortex of patients with Alzheimer's disease (Iwamoto et al., 1991), and 3) the possible involvement of calpain in nerve cell death observed in experimental ischemia and hypoxia (Nilsson et al. 1990; Lee et al., 1991; Ostwald et al., 1993; Rami et al., 1993; Yoshida et al., 1995). Whatever the case, the increased expression of calpain, probably the reflection of an increased intracellular calcium concentration observed in the catecholaminergic cell groups affected in Parkinson's disease, may participate in the mechanism of nerve cell death. This rise in intracellular calcium concentration may provoke deleterious effects, such as the activation of endonucleases and phospholipases. Nevertheless, whether increased m-calpain concentrations represent an early or a late event in the mechanism leading to nerve cell death, and whether they occur earlier than oxidative stress, remains to be established.

Conclusion

The above data suggest that two populations of dopaminergic neurons may exist in the mesencephalon: one population represented by the neurons of the central gray substance, and the other by the dopaminergic neurons of the calbindin-poor zone in the substantia nigra pars compacta. These two populations of neurons are particularly sensitive to oxidative stress and increased calcium concentrations, both of which may occur in Parkinson's disease. The great vulnerability of these two populations of neurons to these deleterious compounds may well explain why they degenerate in the disease.

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Inositol trisphosphate and calcium signalling

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Inositol trisphosphate is a second messenger that controls many cellular processes by generating internal calcium signals. It operates through receptors whose molecular and physiological properties closely resemble the calcium-mobilizing ryanodine receptors of muscle. This family of intracellular calcium channels displays the regenerative process of calcium-induced calcium release responsible for the complex spatiotemporal patterns of calcium waves and oscillations. Such a dynamic signalling pathway controls many cellular processes, including fertilization, cell growth, transformation, secretion, smooth muscle contraction, sensory perception and neuronal signalling.

A DECADE has passed since the first report of a second messenger function for inositol(1,4,5)-trisphosphate ($InsP_3$)[1]. In that time we have found out how it is produced, how it is metabolized and, now that its receptor has been cloned, we are beginning to understand how it acts to release calcium[2-6]. The recent disclosure that Lowe's oculocerebrorenal syndrome is caused by a defect in a gene that encodes one of the enzymes that metabolizes $InsP_3$ (ref. 7) will ensure a continuing interest in this messenger pathway well into the next decade.

It is through its regulation of intracellular calcium that $InsP_3$ functions to regulate so many cellular processes[2,3,5,6]. In response to many stimuli (such as neurotransmitters, hormones and growth factors) both $InsP_3$ and diacylglycerol (DAG) are formed by the hydrolysis of an inositol lipid precursor stored in the plasma membrane. The $InsP_3$ released into the cytoplasm mobilizes calcium from internal stores, whereas DAG activates protein kinase C (ref. 8). This bifurcating messenger system operates throughout the life of a typical cell, beginning with gametogenesis, fertilization, cell proliferation and early development and continuing through differentiation to perform very precise control functions in a whole variety of specialized cells in both animals and plants. Before describing its role in some specific cellular processes, I shall summarize how $InsP_3$ is released from the membrane and how it acts to generate calcium signals.

Signalling pathways and molecular heterogeneity

External signals arriving at the cell engage surface receptors to initiate signalling pathways whereby information flows from one component to the next until the final effector system is activated. The formation of $InsP_3$ is the focal point for two major pathways, one initiated by a family of G protein-linked receptors and the other by receptors linked by tyrosine kinases either directly or indirectly (Fig. 1). These separate receptor mechanisms are coupled to energy-requiring (GTP or ATP) transducing mechanisms which activate phospholipase C (PLC) to hydrolyse the lipid precursor phosphatidylinositol 4,5-bisphosphate ($PtdIns(4,5)P_2$) to give both DAG and $InsP_3$. The latter then binds to an $InsP_3$ receptor (IP_3R) to mobilize stored calcium and to promote an influx of external calcium, perhaps working in conjunction with $InsP_4$ (refs 9, 10).

What is not apparent from Fig. 1 is the rich diversity of the individual components within these signalling pathways. Classical pharmacology prepared us for receptor diversity in that each of the stimuli listed in Fig. 1 acts through a separate receptor. What came as a surprise, however, was the extensive heterogeneity of the downstream elements, such as the G proteins (Box 1), PLC (Box 2), IP_3R (Box 3) and PKC (ref. 8). The full extent of this diversity is apparent in *Drosophila* photoreceptors where the transducing elements rhodopsin, DG_q and norpA (Box 1) are present primarily in the eye. Why did this diversity arise? We can only surmise that a few basic signalling systems emerged early in evolution and were then modified in subtle ways to meet the unique signalling requirements of different cells. In order to detect brief flashes of light, photoreceptors face very different problems from liver cells responding to slower changes in circulating hormone levels. As cells differentiate to perform separate functions, they select out specific components from a diverse signalling repertoire to construct personalized messenger pathways precisely adapted to suit their particular requirements. Despite all this molecular heterogeneity, the common theme running through all these variations is that external signals use the $InsP_3$/calcium and DAG/PKC pathways to regulate a wide range of cellular activities.

G protein-linked receptors

The membrane-transducing unit controlled by G protein-linked receptors has three main components—the activated receptor communicates through a G protein to stimulate PLC (Fig. 1 and Box 1). Most of the G protein-linked receptors identified so far are characterized by having seven membrane-spanning domains connected by extracellular and intracellular loops (Box 1). The transmembrane columns interact with each other to form a pocket where the agonist binds to induce the conformation responsible for activating the G protein, that is, the next component of the signalling pathway. This G protein family can be divided into two, depending on whether or not they are sensitive

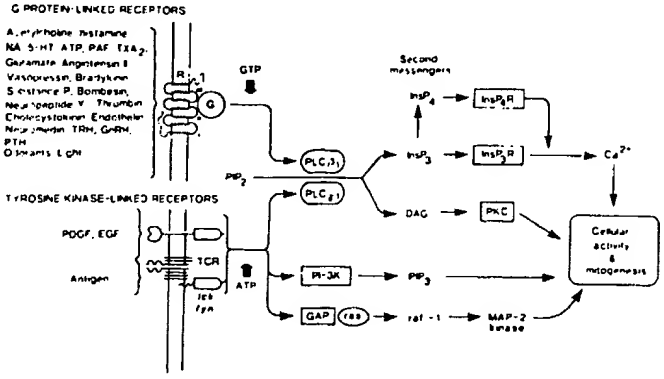


FIG. 1 Summary of the two major receptor-mediated pathways for stimulating the formation of inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG). Many agonists bind to 7-membrane-spanning receptors (R), which use a GTP-binding protein (G) to activate phospholipase C-β1 (PLC-β1), whereas PLC-γ1 is stimulated by the tyrosine kinase-linked receptors (see Box 2). The latter activate other effectors such as the phosphatidylinositol 3-OH kinase (PI-3K), which generates the putative lipid messenger phosphatidylinositol (3,4,5)-trisphosphate (PIP_3) and the GTPase-activating protein (GAP) that regulates *ras*. $InsP_3R$, $InsP_3$ receptor; PKC, protein kinase C; NA, noradrenaline; 5-HT, 5-hydroxytryptamine; PAF, platelet-activating factor; TXA_2, thromboxane A_2; TRH, thyrotropin-releasing hormone; GnRH, gonadotrophin-releasing hormone; PTH, parathyroid hormone.

to pertussis toxin. The second and third cytoplasmic loops of the receptor (see Box 1 for details) has an essential role in activating a member of the heterotrimeric G-protein family[17] responsible for stimulating separate members of the PLC family (Box 1)[18,19].

Tyrosine kinase-linked receptors

The other pathway responsible for stimulating the release of $InsP_3$ begins with tyrosine kinase receptors, which relay information through a direct interaction between the receptor and the γ-form of PLC (Fig. 1 and Box 2). As for the G protein-linked receptors, signal transduction through tyrosine kinase receptors is an energy-requiring process because ATP is consumed not only as the two receptors interact (autophosphorylation), but also during the subsequent phosphorylation of PLC-γ_1 (Box 2). Growth factors such as platelet-derived (PDGF) and epidermal (EGF) growth factors act by bringing two receptors together, which enables their cytoplasmic kinase domains to phosphorylate each other on tyrosine residues to create docking sites to bind PLC-γ_1. In unstimulated cells, PLC-γ_1 is largely cytosolic but translocates to the membrane as its SH2 domain binds to the activated receptor (Box 2). This association has two important consequences for the activation of PLC-γ_1, it is phosphorylated by the receptor on specific tyrosine residues and its membrane translocation brings it into contact with its substrate PtdIns(4, 5)P_2. A similar activation process is responsible for stimulating PLC-γ_1 following crosslinking of IgM receptors in B lymphocytes or activation of the T-cell antigen receptor (TCR)–CD3 receptor complex of T cells. These lymphocyte receptors lack tyrosine kinase activity but they recruit members of the *src* family such as *fyn* and *lck* (Fig. 1).

The dynamics of different transducing mechanisms have been compared in NIH 3T3 cells, which carry both a G protein-linked receptor (bradykinin) and a tyrosine kinase-linked receptor (PDGF)[24]. When compared to bradykinin, the PDGF-induced formation of $InsP_3$ was much slower, and the resulting calcium response was not only smaller but had a much longer latency. Following transfection with a complementary DNA for PLC-γ_1, the effects of bradykinin were unchanged, whereas the PDGF-induced responses were markedly enhanced; the rate of $InsP_3$ formation was increased, resulting in a larger calcium signal with a reduced latency.

$InsP_3$ and ryanodine receptors

$InsP_3$ and ryanodine receptors (RYRs) represent the two principal intracellular calcium channels responsible for mobilizing stored calcium. These two receptors will be considered together because they share considerable structural and functional homologies[5,25]. A family of IP_3Rs has now been identified (see Box 3)[26,27] with molecular diversity arising from both alternative splicing and the existence of separate genes[28,29]. The IP_3R contains typical membrane-spanning domains in the C-terminal region which anchor the protein in the membrane with four of the subunits combining to form the functional $InsP_3$-sensitive calcium channel (Fig. 2*c*). The large N-terminal domain lies free in the cytoplasm with the $InsP_3$ binding site located at its end, a long way away from the channel-forming C-terminal region[30]. Upon binding $InsP_3$, the receptor undergoes a large conformational change which is perhaps related to the coupling process leading to channel opening[30].

The other major intracellular calcium channel, the RYR, has three family members (Box 3). The RYRs identified in neurons[41] and sea urchin eggs[42] seem to resemble the cardiac RYR2. Some cells respond to transforming growth factor (TGF)-β by expressing a much smaller RYR3 (ref. 34). Cells carrying RYR3 were insensitive to caffeine and ryanodine but the latter did abolish the effects of bradykinin, suggesting that RYR3 and IP_3R are co-localized. Like the IP_3R, RYRs exist as tetramers, with the C-terminal regions co-operating to form the calcium channel, and the large N-terminal regions forming bulbous heads that project into the cytosol (Fig. 2*a* and *b*). The two ways in which these RYRs respond to membrane depolarization is illustrated in Fig. 2. The plant alkaloid ryanodine opens the channels at low (nanomolar) concentrations but closes them at higher doses

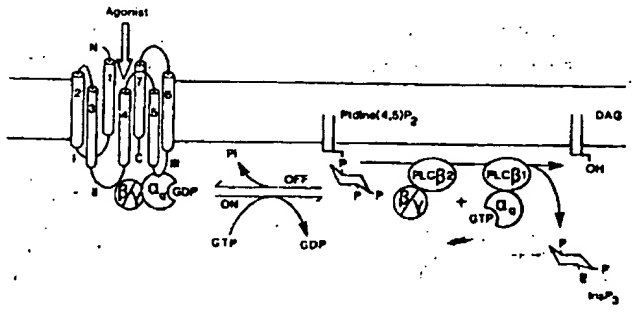
BOX 1 Signal transduction through G protein-linked receptors

Receptor–G protein–phospholipase C transduction unit

THE on-reaction begins when the agonist induces a conformational change in the receptor which is transmitted to the G protein loops II and III. The heterotrimeric G proteins dissociate into G_α and $G_{\beta\gamma}$ subunits, both of which can activate different PLC isozymes (see Box 2). The G_α subunits exchange GDP for GTP before interacting with P and G sites on the C-terminal region of PLC-β1 (ref. 11; see Box 2). There is growing evidence that the $\beta\gamma$ subunit may also play a role[12,13], particularly to activate PLC-β2 (ref. 13). This membrane-transducing unit can be activated experimentally by injecting cells with GTP-γS or by treating them with fluoride. The venom mastoparan can also activate by mimicking the molecular interaction operating between the receptor and the G protein.

Phospholipase C functions as a GTPase-activating protein

During the off-reaction, GTP is hydrolysed to GDP by the GTPase associated with the α-subunit which then re-combines with $\beta\gamma$ to form an inactive complex. $G_{\alpha q}$ has a very low intrinsic GTPase activity which is greatly enhanced following its interaction with PLC-β1, suggesting that the latter functions like the GTPase-activating protein (GAP) which associates with the proto-oncogene $p21^{ras}$ (ref. 14). This finding that the effector molecule PLC-β1 functions like GAP increases the likelihood that the latter might mediate the effects of $p21^{ras}$.



Receptors	G protein subunit	PLC
Pertussis toxin-sensitive		
Glutamergic (mGlu)	$G_{\alpha o}$	?
Pertussis toxin-insensitive		
Rhodopsin (fly)	$DG_{\alpha q}$	norpA
Muscarinic (M1, M3 and M5)		
Serotonergic (5-HT1c)	$G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$	PLC-β1
Adrenergic (α1B)	$G_{\alpha 16}$	PLC-β2
	$G_{\beta\gamma}$	PLC-β2

See Fig. 1 for further examples of pertussis toxin-insensitive receptors.

The role of cytoplasmic loops in coupling receptors to G proteins

Mutations in the third cytoplasmic loop of the adrenergic-1B receptor resulted in a constitutive activation of PLC, presumably by mimicking the active conformation normally induced by the agonist[15]. Substitution of alanine at position 293 (located in loop III close to transmembrane domain 6) with any of the 19 possible alternative amino acids all resulted in a constitutive increase in PLC activity. The wild-type receptor therefore has evolved a loop structure which gives the lowest basal activity. Transfection of cells with mutant receptors showing the highest constitutive activity results in transformation and tumorigenesis[16].

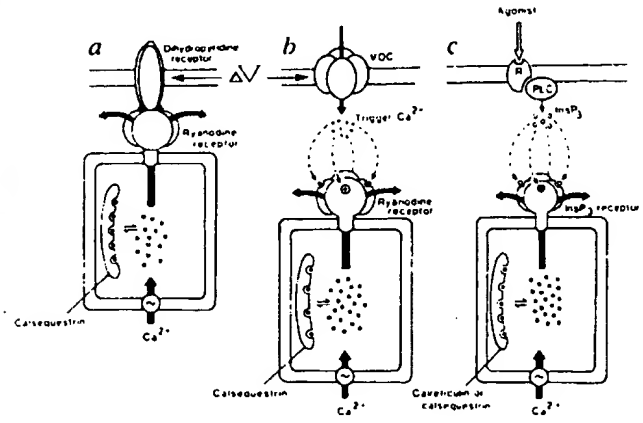


FIG. 2 Control of calcium release by intracellular tetrameric calcium channels. *a*, RYRs located in the sarcoplasmic reticulum of skeletal muscle contribute to the T-tubule foot structure responsible for excitation-contraction coupling. The dihydropyridine receptor in the surface membrane senses a change in voltage (ΔV) and undergoes a conformational change which is transmitted through the bulbous head of the RYR to open the calcium channel in the sarcoplasmic reticulum. *b*, Calcium-induced calcium release in cardiac muscle and perhaps also in neurons. A voltage-operated channel (VOC) responds to ΔV by gating a small amount of trigger calcium, which then activates the RYR to release stored calcium. *c*, Agonist-induced calcium release. Signal transduction at the cell surface generates $InsP_3$ which diffuses into the cell to release calcium by binding to IP_3Rs (see Box 3).

(>micromolar). Caffeine can also open the RYR channel. These RYRs contribute to calcium signalling in many different cell types (skeletal muscle, cardiac muscle, neurons, chromaffin cells, smooth muscle, pituitary cells and sea urchin eggs).

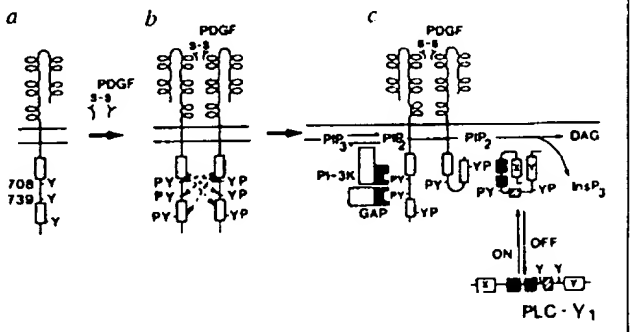
The remarkable structural similarity that exists between IP_3Rs and RYRs probably reflects a common evolutionary origin which is supported by the very close sequence homology identified primarily in the two membrane-spanning domains and in the free C-terminal region, which includes two cysteines within a TXCFICG motif that is absolutely invariant for all the intracellular calcium channels examined so far (Box 3). This free C-terminal tail may play some part in channel opening because monoclonal antibodies that bind to this region (see Box 3) can either inhibit[35] or enhance $InsP_3$-induced calcium release[36]. The conserved cysteine residues located in this region may also represent the target sites of thiol reagents which enhance the calcium-mobilizing activity of both receptor families (see later). This structural and molecular homology may account for many of the functional similarities that exist between IP_3Rs and RYRs.

Intracellular calcium stores

The membrane stores from which calcium is released contain three major components—pumps to sequester calcium, binding proteins (such as calsequestrin and calreticulin) to store calcium and the specific IP_3R or RYR channels to release calcium back into the cytosol (Fig. 2). These receptors are normally located on modified portions of the endoplasmic reticulum (ER)[43]. One such structure, the calciosome, was originally thought to be the $InsP_3$-sensitive store, but recent studies on Purkinje neurons suggest that these organelles may be heterogeneous as some may be sensitive to ryanodine[44]. The distribution of such $InsP_3$- or ryanodine-sensitive calcium stores varies considerably from cell to cell. Some cells either have ryanodine-sensitive stores (skeletal muscle) or $InsP_3$-sensitive stores, as in *Xenopus*

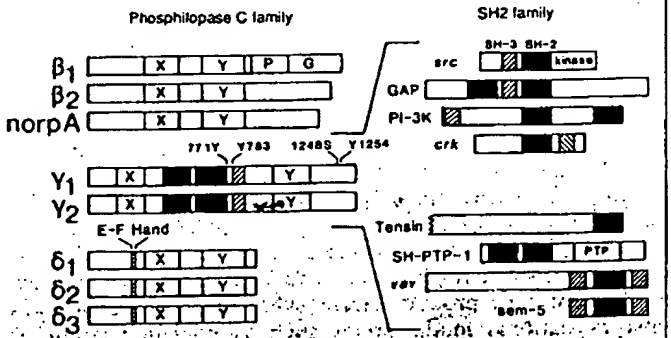
BOX 2 Signal transduction through tyrosine kinase-linked receptors

TYROSINE kinase receptors (such as PDGF, EGF) generate $InsP_3$ and DAG by interacting directly with PLC-γ1, one of the PLC family members (see below). The sequence of events is illustrated using the PDGF receptor as an example. *a*, The receptor consists of a single transmembrane protein containing a cytoplasmic tyrosine kinase which is separated into two (boxes). *b*, PDGF induces dimerization allowing the two kinase domains to phosphorylate each other on specific tyrosine residues (Y) which provide docking sites responsible for interacting with different members of the SH2 family. *c*, An SH2 domain on PLC-γ1 (filled boxes) recognizes and binds to a specific phosphotyrosine residue. Once the PLC-γ1 is phosphorylated on specific residues (see below) it begins to hydrolyse PtdIns(4,5)P_2 (labelled here as PIP_2) to give DAG and $InsP_3$.



There are three major PLC family members (β, γ, δ)[18,19]. An earlier report of a cDNA coding for a PLC-α (ref. 20), which showed no sequence identity to the other PLC enzymes, may not be a PLC but is probably thiol-protein disulphide oxidoreductase[21]. There is no sequence data on a new PLC-ε family[19]. Two sites (P and G) on PLC-β1 are responsible for interacting with $G_{\alpha q}$[11]. There is little sequence homology between PLC-β, PLC-γ and PLC-δ, except for domains X (~170 amino acids) and Y (~260 amino acids). PLC-β and norpA are coupled to G proteins (Box 1), whereas PLC-γ1 interacts with tyrosine kinase-linked receptors (see above) which phosphorylate the enzyme on at least 3 tyrosines (771, 783 and 1,254) that are important for activating the enzyme. Phosphorylation of the serine (S) at 1,248 by PKC or PKA may be responsible for inhibiting signal transduction[18]. The mode of activation of PLC-δ is unknown. It may represent a calcium-sensitive form of the enzyme as it contains one canonical EF-hand motif[22]. The activity of PLC-δ_1 is specifically increased in the aortas of spontaneously hypertensive rats[23].

The *src* proto-oncogene contains two domains, called *src* homology 2 and 3 (SH2 and SH3), which are found within the structure of many other proteins, particularly those concerned with signal transduction. PLC-γ is also a member of this family as it contains two SH2 domains (filled boxes) and one SH3 domain (hatched box). A new family member is a phosphotyrosine phosphatase (SH-PTP-1) containing two SH2 domains. The



SH2 domains function as adaptors that bind to specific tyrosine residues (see above). Located near each phosphorylated tyrosine there is a short sequence motif which determines the specificity of binding to various members of the SH2 family.

oocytes[45], whereas there are others that contain both (atrial cells, vascular smooth muscle, neurons, chromaffin cells and sea urchin eggs). Cerebellar Purkinje cells have both RY and IP_3Rs distributed throughout the cytosol on elements of the smooth endoplasmic reticulum (ER) in both the soma and along the dendrites[41,46]. Unlike the RYR, however, the IP_3R is also found in the spines[41]. In double-labelling experiments, some elements of the endoplasmic reticulum had both receptors. Although there is some physiological evidence to support such a co-localization in some cell types such as PC12 cells[47], most studies indicate that these two receptors operate separate stores, as in sea urchin eggs[48]. However, these two stores may interact with each other to generate calcium signals.

$InsP_3$-induced calcium release

Calcium contained within intracellular stores is released to the cytosol when $InsP_3$ binds to its receptor (Fig. 2*c*)[2,4-6]. This action of $InsP_3$ has been studied either at the single receptor level, in single cells or in cell populations. When embedded in a lipid membrane, the IP_3R responds like a conventional channel, displaying an increase in open frequency in response to $InsP_3$ (refs 49, 50). The mean open time was less than 10 ms and there was evidence of four conductance states, each of ~20 pS (ref. 50). On the basis of finding evidence of co-operativity (Hill coefficients (n_H) ≥ 3), Meyer *et al.*[51] proposed that channel opening depends upon $InsP_3$ binding sequentially to the four putative binding sites of the tetrameric receptor (Fig. 2*c*). Each binding step could cause a partial opening of the channel in order to account for the different conducting states observed in patch recordings of purified IP_3Rs (ref. 49). However, others have not found any evidence of co-operativity ($n_H = 1$)[50,52] suggesting that binding of a single molecule of $InsP_3$ is sufficient to account for channel opening. Clearly we still have a long way to go to understand just how $InsP_3$ acts to open individual channels.

At the next level of organization, the action of $InsP_3$ has been studied on populations of receptors prepared either in membrane vesicles or in their normal location within permeabilized cells. The response is extremely fast, reaching a maximum within 140ms of adding $InsP_3$ to synaptosomes[52]. All these studies have revealed considerable variability in the sensitivity of $InsP_3$-induced release, which gives rise to the phenomenon of 'quantal calcium release'[53-56]. As the level of $InsP_3$ rises, a fixed proportion of the stored calcium is released, with the remainder becoming accessible at higher doses. This variability is evident when studying purified receptors in artificial membranes[56], calcium release in single cells[55,57] or in cell populations[53-55]. A spatial analysis of calcium release triggered by applying increasing doses of $InsP_3$ to *Xenopus* oocytes revealed the existence of hot spots, confirming that the $InsP_3$-sensitive stores were not uniformly sensitive[57].

There are two main ways in which the sensitivity of $InsP_3$-induced calcium release might vary. First, IP_3R sensitivity may change depending on the calcium content of the endoplasmic reticulum[38,58-60]. The available evidence suggests that its sensitivity increases as the store charges up with calcium. As there are no obvious calcium-binding domains within those parts of the IP_3R that face the ER lumen, the calcium-sensing might be mediated by a separate protein with calsequestrin or calreticulin (Fig. 2) being obvious candidates. However, such a mechanism cannot explain the apparent quantal release of calcium by purified IP_3Rs reconstituted into liposomes[56]. The second proposal, therefore, is that variations in sensitivity may depend upon receptor heterogeneity (Box 2) arising from the presence of different gene products, from alternative splicing or from post-translational modifications such as phosphorylation or autophosphorylation (because the IP_3R can function as a protein kinase[61]). Phosphorylation of the unspliced neural version results in a decrease in sensitivity[62], whereas the opposite occurs for the spliced version found in the periphery[63]. As individual cells can express different IP_3Rs, variations in sensitivity might arise by varying the contribution of different subunits to the tetrameric receptor complex[29].

At the heart of the current debate, therefore, is the question of what determines the different sensitivities of IP_3Rs. Is it a difference in receptor structure or is the variability imposed upon the receptor by the calcium content of the endoplasmic reticulum? It is essential that we resolve this question because it is relevant not only to the process of calcium entry but also to the mechanism of calcium spiking in intact cells (see later).

Calcium-induced calcium release

The considerable structural and molecular homology shared by RYR and IP_3R is mirrored in many functional similarities, the most striking of which is a common sensitivity to calcium. Each heartbeat is driven by a regenerative process of calcium-induced calcium release (CICR), when a small influx of calcium through voltage-operated calcium channels triggers an explosive release of stored calcium from the sarcoplasmic reticulum (Fig. 2*b*). This positive feedback process whereby calcium triggers its own release is a property of RYRs and it may also occur in those non-muscle cells expressing these receptors. As in the heart, calcium entering neurons through voltage-operated channels can be amplified by CICR from internal pools[64] and this can lead to the generation of repetitive calcium spikes[65]. RYRs may also generate calcium spiking in mouse eggs[66] and pancreatic acinar cells[67]. This calcium-sensitive regenerative property of RYRs is also displayed by IP_3Rs.

Perhaps the most interesting property of $InsP_3$-induced calcium mobilization is its all-or-none property manifested as a sudden and near-maximal release of calcium if the level of $InsP_3$ is gradually increased through injection[68] or by flash photolysis of caged $InsP_3$ (ref. 57). When visualized by confocal microscopy, the response in *Xenopus* oocytes had two phases. Initially there were small hot spots resulting from the localized release of calcium, which is probably related to the phenomenon of quantal calcium release described earlier. Above a certain threshold, these hot spots suddenly transformed into the all-or-none response that appeared as expanding circular or spiral waves[57,69,70]. This all-or-none response seems to arise through a positive feedback effect whereby calcium stimulates its own release. The IP_3R displays a bell-shaped response to calcium which thus functions as a coagonist with $InsP_3$ to release stored calcium[52,71,72]. In the absence of calcium, $InsP_3$ has little effect, but becomes increasingly active as the concentration of calcium rises, reaching a maximum at about 300 nM, after which it begins to be inhibitory. Using flash photolysis of caged $InsP_3$ and caged calcium, Iino and Endo[73] demonstrated that these positive and negative effects of calcium operated sequentially during the rising phase of a calcium spike. Positive feedback enhances the initial release, but this is soon curtailed as the accumulation of calcium activates the negative feedback component. The latter may depend upon the receptor switching from a low-affinity (R_L) state, which can gate calcium, to an inactive high-affinity (R_H) state[74]. Once the concentration of calcium returns to its resting level, the inactive R_H state converts back to the sensitive R_L state in about one second, thus setting the stage for another spike. This calcium-dependent interconversion of the IP_3R may contribute to the mechanism of repetitive calcium spiking[52,73,74] (see later). Like the RYR, therefore, the IP_3R also seems to have a CICR mechanism which is of fundamental importance for understanding of how cells generate calcium oscillations and waves.

Inositol phosphates and calcium influx

Calcium entry into cells can be regulated by a number of mechanisms, for example through channels operated by voltage, by receptors or by second messengers. With regard to the latter, most attention has focused on the inositol phosphates, $InsP_3$ and $InsP_4$ (refs. 9, 10). In some cells, there are suggestions that these inositol phosphates may directly activate specific channels

in the plasma membrane. For example, the $InsP_3$-induced entry of calcium in lymphocytes may be mediated by a new IP_3R which contains sialic acid and is localized in the plasma membrane[75]. Similarly, the plasma membrane of olfactory cells seems to have an $InsP_3$-sensitivie calcium channel[36,76,77]. When compared with the IP_3Rs on the ER, putative plasma membrane receptors are less specific with regard to $InsP_4$ (refs 75, 76). In olfactory cells, for example, the binding protein was equally sensitive to $InsP_3$ and $InsP_4$ (ref. 76). By contrast, the $InsP_4$-sensitive calcium channel in the plasma membrane of endothelial cells was insensitive to $InsP_3$ (ref. 78). There is growing evidence, therefore, that inositol phosphates may have direct effects on calcium channels within the plasma membrane.

In addition to these direct effects, $InsP_3$ may stimulate calcium entry indirectly through a more complex mechanism involving the ER. Putney[79] coined the term 'capacitative entry' to introduce the idea that the influx of external calcium seemed to be regulated by the calcium content of a portion of the ER lying close to the plasma membrane. In some cells, calcium entry is stimulated when the ER stores are artificially emptied by applying the calcium pump inhibitors thapsigargin[47,80-82] or 2,5-di-*tert*-butylhydroquinone[82], or the calcium ionophore ionomycin[81]. When the ER is fully charged, entry is prevented, but as soon as $InsP_3$ drains calcium out of these stores, the influx of calcium switches on automatically. Such a mechanism is thought to account for the entry of calcium into lacrimal[80] or mast cells[81] following cell perfusion with $InsP_3$. Others, also using lacrimal cells, failed to see such an $InsP_3$-dependent entry unless they added $InsP_4$ (ref. 83). Irvine[10] has presented a detailed analysis of the controversy regarding the role of these two inositol phosphates in regulating calcium entry.

The most difficult aspect to explain concerning this capacitative mechanism is how the calcium content of the ER determines the rate of calcium entry across the plasma membrane. An interesting possibility is that the IP_3R might function to communicate information between the ER and the plasma membrane[9,84]. By analogy with excitation-contraction coupling, in which the large cytoplasmic head of the RYR links the T-tubule to the internal calcium store (Fig. 2*a*), the large head of the IP_3R may also convey information between the ER and the plasma membrane[9,84]. The idea is that this cytoplasmic head communicates with two calcium channels, one in the ER and the other in the plasma membrane. $InsP_3$ binding will induce a conformational state that opens the ER channel, and as the pool loses its calcium, the receptor may change its conformation, leading to an opening of the plasma membrane channel. Irvine[9,10] has argued that the opening of this entry channel may also require $InsP_4$ and that such an action would explain those instances where both inositol phosphates are required. As yet there is no information on whether stores regulated by RYRs can induce capacitative entry if their calcium is drained by agents such as caffeine.

In summary, the conformational coupling hypothesis considers that the IP_3R controls the mobilization of both internal and external calcium. Although the mechanism of calcium entry remains a matter for debate, what is not in doubt is that elevated levels of $InsP_3$, perhaps acting together with $InsP_4$, can maintain a constant influx of calcium. In cells that are oscillating, the magnitude of this influx component through second messenger-operated channels is often rather small and has little effect on the intracellular level of calcium during the interval between spikes, mainly because it is rapidly sequestered by the internal stores. But by charging up these stores, the influx mechanism transforms the cytoplasm into an 'excitable medium'[69], thus setting the stage for the generation of the repetitive calcium spikes and waves described below. In effect, these internal stores integrate this small influx over time before periodically releasing the accumulated signal as a regenerative calcium spike.

Spatiotemporal aspects of Ca^{2+} signalling

Now that we are beginning to understand the basic mechanisms of calcium release and entry, the next challenge is to describe the complex spatiotemporal patterns of calcium signalling revealed to us by single-cell imaging techniques. Many of the cells that respond to calcium-mobilizing agonists display a repetitive pattern of calcium spikes whose frequency is sensitive to both agonist concentration and the level of external calcium[2,25,84,85]. In addition to this temporal patterning, each spike often displays a recurring spatial organization. There is a specific initiation locus from which the calcium spreads as a regenerative wave[57,67,69,86]. Secretagogue-induced waves in pancreatic acinar cells initiate in the apical region before propagating towards the basal pole[67].

Waves propagate through the cytosol using either ryanodine or IP_3Rs. In sea urchin eggs, the fertilization-induced wave spreads around the periphery (Fig. 3)[87], which corresponds to the cortical location of the RYRs[42]. RYRs are also thought to contribute to wave propagation in pancreatic acinar cells[67] and in mouse eggs[66], whereas IP_3Rs seem to operate in *Xenopus* oocytes[70], *Xenopus* eggs[88] and in hamster eggs[35]. In *Xenopus* oocytes that lack RYRs[45], there are numerous initiation foci (hot spots) and the waves migrate outwards as expanding spheres or spirals which annihilate as they crash into each other[57,69,70,88]. Such spiral waves could be induced by injecting the non-metabolizable analogue $InsPS_3$ (ref. 70), whereas the injection of calcium had no effect even though it caused a localized elevation of calcium[88]. In the presence of $InsPS_3$, however, the injection of calcium was able to initiate waves. Heparin, which is a specific inhibitor of the IP_3R, was found to stop the waves in both *Xenopus*[88] and mouse oocytes[89]. The calcium waves in hamster eggs induced by fertilization or a local injection of $InsP_3$ were inhibited by prior injection with the antibody that binds to the C-terminal region of the IP_3R (Box 3)[35]. Although all this evidence suggests a central role for the

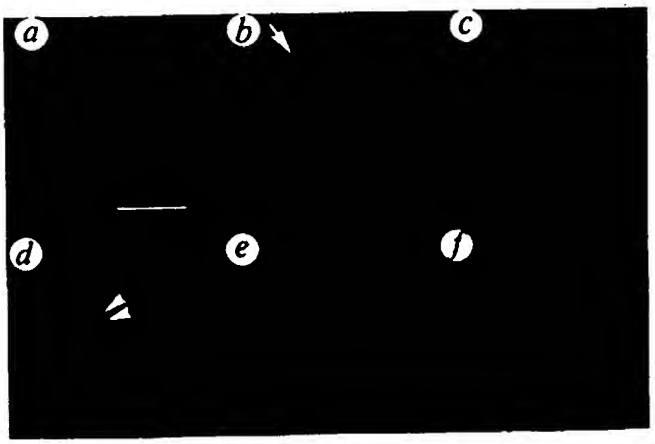


FIG. 3 Fertilization calcium wave in a sea urchin egg observed by confocal microscopy using the fluorescent-indicator fluo-3. The arrow (*b*) illustrates the initiation point of the calcium wave, which then sweeps around the periphery of the cell (frames *c*–*f*). The wave propagated at a rate of 3–10 $\mu m\ s^{-1}$. Note the large increase in nuclear calcium (arrowheads in *d*). (Reproduced with permission from Stricker *et al.*[87].)

IP_3R, at least in certain cells, there is controversy concerning the way in which it contributes to the initiation and propagation of calcium waves.

Many ingenious models have been proposed to explain these spatiotemporal aspects of calcium signalling[2,25,84,85]. The emerging consensus is that the basic mechanism requires an element of positive feedback whereby calcium amplifies its own release. One view is that calcium exerts a positive feedback effect on PLC, thereby generating periodic surges of $InsP_3$ during each spike[85]. In this cross-coupling model, the wave propagates by $InsP_3$ diffusing from one store to the next, with calcium-induced $InsP_3$ formation acting to maintain a steep diffusion gradient for $InsP_3$. The alternative view is that calcium is the diffusible messenger that enhances its own release through the process of CICR (Fig. 4).

This generalized CICR model has two main components (Fig. 4). An entry mechanism at the plasma membrane, which regulates the supply of calcium to the second component, the oscillator itself, which consists of the calcium stores that have either the RYRs or IP_3Rs responsible for CICR. The nature of the entry mechanism can also vary: in neural cells it is through voltage-operated channels, whereas in other cells it is usually mediated by the $InsP_3$-dependent entry mechanisms described earlier. In the latter case, where entry depends upon a capacitative mechanism based on an $InsP_3$-sensitive pool, the model shown in Fig. 4 transforms into the two-pool model described in detail elsewhere[84,90]. The crux of this model, which can be divided into four distinct phases (Fig. 4*a–d*), is that repetitive spiking depends upon the cyclic release of calcium from internal stores through the regenerative process of CICR. A critical factor appears to be the enhanced influx of external calcium which is taken up by the stores with two consequences. First, the build up of calcium may serve to sensitize the receptor as discussed earlier. A beautiful demonstration of the importance of calcium loading in priming RYRs was described in sympathetic ganglion neurons, where the rapid removal of external calcium could prevent the caffeine-induced spikes right up to the onset of the all-or-none phase (Fig. 4*c*)[65]. Second, as the stores fill up, their buffering capacity will be reduced, thus leading to a pacemaker elevation of cytosolic calcium (Fig. 4*b*), which often appears at

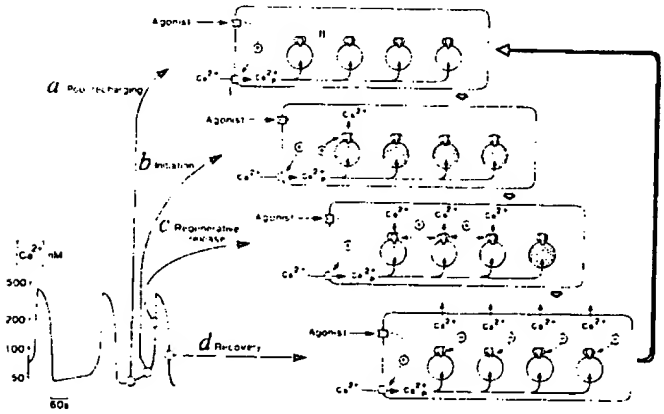


FIG. 4 Calcium-induced calcium release (CICR) model of calcium spiking. The trace on the left represents a typical baseline spiking pattern very similar to that described in cells such as mammalian eggs[66,68] or sympathetic neurons[65]. The model has two main components, calcium entry (I) and the internal stores (II) carrying IP_3Rs or RYRs, depending on the cell type. *a*, One of the functions of the external stimulus is to promote an entry of external calcium, often mediated by $InsP_3$, to give the primer calcium (Ca^{2+}_p) which charges up the internal stores. *b*, Once these stores are loaded, a process of CICR begins to activate either the IP_3Rs or RYRs to release calcium. *c*, Calcium functions as a messenger to release calcium from neighbouring stores to propagate a wave. *d*, When release ceases, probably owing to a negative feedback effect of calcium, recovery occurs as calcium is pumped out of the cytoplasm (by pumps on the plasma membrane and internal stores) and the cell is ready to begin another cycle.

a specific initiation site where it provides the trigger to detonate the process of CICR (Fig. 4, step c). These two components were clearly demonstrated in *Xenopus* oocytes, in which the response to gradually increasing doses of $InsP_3$ began with localized elevations of calcium (hot spots) which then transformed into regenerative waves[57]. Dissociation of initiation from propagation probably accounts for abortive spikes such as those observed in HeLa cells responding to low doses of histamine[55]. These initiation sites may represent areas containing calcium stores that are particularly sensitive to activators such as $InsP_3$. Finally, the spike terminates through a combination of events. The build up of cytosolic calcium inhibits further release through its inhibitory effect on the release channel and the cytosolic calcium is removed by being pumped back into the stores or out of the cell (Fig. 4*d*). By measuring the efflux of calcium into a microdroplet surrounding single pancreatic cells, Tepikin *et al.*[91] have found that 15–70% of the calcium spike is extruded from the cell during the recovery phase.

A critical part of this model concerns the change in receptor sensitivity which sets the stage for the initiation of the spike by CICR. Variations in receptor sensitivity could also account for variations in both spike amplitude and rates of wave propagation (M.D. Bootman, personal communication). For example, wave propagation rates in pancreatic acinar cells increased from 57 to 95 $\mu m\ s^{-1}$ when the concentration of acetylcholine was raised from 0.1 to 1.0 μM[67]. Lower stimulus intensities will engender fewer sensitized stores and the greater diffusional distances will slow down the wave speeds. Both RYR and IP_3R display variations in sensitivity. For example, caffeine-induced spiking in neurons results from a lowering of the threshold for CICR. The CICR mechanism evident in eggs at fertilization may be sensitized by a sperm factor[66]. A possible candidate is the cyclic ADP ribose (cADPR)[48] which is formed from NAD^+ (ref. 92). When added to sea urchin egg homogenates, cADPR released calcium from the same stores operated by RYRs. A subthreshold dose of cADPR also sensitized homogenates to the action of ryanodine and caffeine[48]. Just how cADPR acts to sensitize the RYR remains to be shown. A similar change in sensitivity has been described for the $InsP_3$-sensitive calcium channels in permeabilized liver cells, which spontaneously released their calcium when the stores were overloaded[58]. During the loading period, the responsiveness of the IP_3Rs gradually increased until they were able to respond to the ambient level of $InsP_3$. IP_3R sensitivity can also be enhanced by specific thiol reagents, such as oxidized glutathione or thimerosal[58,93,94], which probably explains how these agents induce calcium spiking in intact hepatocytes[95], mammalian eggs[96,97] and HeLa cells[94]. The thimerosal-induced calcium spiking in hamster eggs was blocked by the antibody 18A10 (Box 3), confirming the notion that these thiol reagents are acting through the IP_3R (ref. 97). $InsP_3$-sensitive calcium stores can thus operate in two separate modes. They can either respond to a rise in the level of $InsP_3$, or they can release their calcium in the presence of a constant level of $InsP_3$ through a process of CICR.

In summary, when cells are activated, the cytoplasm becomes an excitable matrix which allows a calcium signal to initiate periodically at a specific point before spreading throughout the cell as a regenerative calcium wave. This spatiotemporal organization of calcium signalling seems to depend upon two key processes—influx of calcium across the plasma membrane and a regenerative release of calcium from internal stores controlled by either RYR or IP_3R.

Intercellular calcium waves

Waves are not confined to single cells but can travel from cell to cell through two separate mechanisms. In cells lacking gap junctions (clusters of leukaemia cells, for example), waves spread by means of a secreted intermediate (ATP)[98]. The alternative mode of transmission is through gap junctions as described in ciliated epithelial cells of the lung[99] or in glial cell populations

both *in vitro*[100] and *in situ*[101]. Just how the wave crosses the gap junction is unknown, but it could depend upon the diffusion of either calcium itself or $InsP_3$. As the wave reaches the cell periphery, enough calcium may diffuse across to activate the neighbouring cell. Alternatively, the cross-coupling model[85] described earlier would predict a localized calcium-activated elevation of $InsP_3$, which could then diffuse across the gap junction[99]. In the case of *Xenopus* oocytes, there is indirect evidence that $InsP_3$ generated in the surrounding follicle cells in response to angiotensin II diffuses across gap junctions to stimulate calcium release in the oocyte[102]. The calcium waves spreading through glial cells may constitute a long-range signalling network acting in concert with conventional neuronal networks. On a more speculative note, an interaction between glial and neuronal oscillators may contribute to the circadian time-keeping mechanism located within the suprachiasmatic nucleus[103].

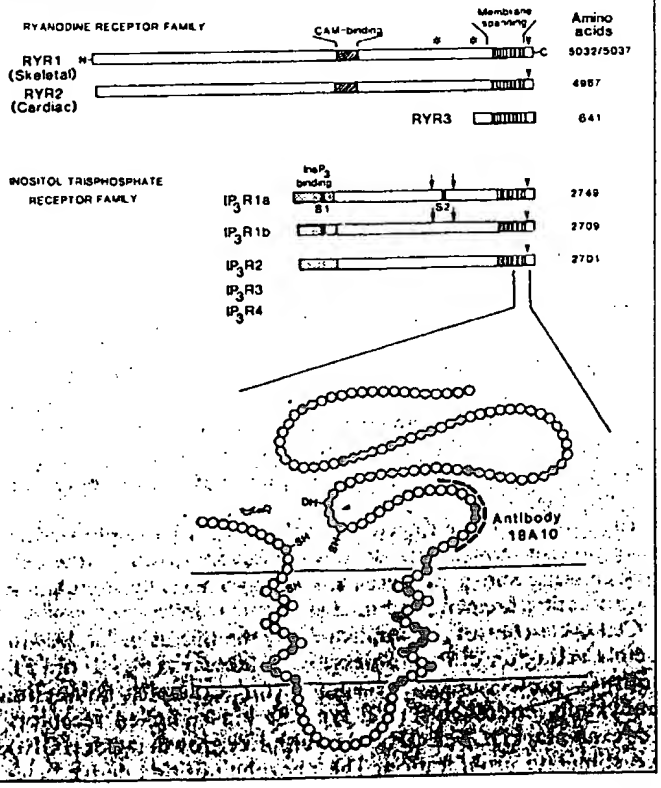
Fertilization and development

The phosphoinositide signalling system is fully established in the gametes and is called upon to regulate major events throughout the life history of a typical cell. $InsP_3$ does not appear to play any part in the maturation of invertebrate and amphibian eggs, but it may contribute to the spontaneous maturation of bovine oocytes[104]. The ability of cyclic AMP to inhibit maturation can be overcome by injecting $InsP_3$. During spontaneous maturation, mouse oocytes display $InsP_3$-dependent repetitive calcium spikes for at least two hours, often extending beyond the point of germinal vesicle breakdown[89]. Although the available evidence argues against a role in germinal vesicle breakdown, these calcium spikes might control associated events such as cytoplasmic maturation[89]. Once eggs have matured they remain in suspended animation, awaiting fertilization when the sperm triggers the wave-like surge of calcium (Fig. 3) responsible for stimulating maturation-promoting factor to activate the developmental programme[105]. Just how the sperm triggers the explosive release of calcium in the egg is still something of a mystery. At the time of fertilization there is an increase in $InsP_3$, and injection of this second messenger can induce some of the early events of fertilization, including the repetitive spiking patterns recorded in mammalian eggs[96,106]. The arguments supporting a role for the IP_3R in propagating calcium waves through *Xenopus* oocytes[70,88] and hamster eggs[97] were summarized earlier. Arguments against a role for a G protein-linked mechanism for generating $InsP_3$ have come from experiments in which phorbol esters failed to block fertilization but could inhibit the events activated by injecting GTP-γS into hamster eggs[106]. An alternative possibility is that the sperm may activate PLC-γ_1 through a tyrosine kinase-linked mechanism similar to that used by growth factors (Fig. 1). Studies on sea urchin eggs seem to argue against a role for $InsP_3$ in fertilization. When injected with heparin there was a normal fertilization-induced calcium transient, whereas the response to either GTP-γS or $InsP_3$ was inhibited[107]. Alternative candidates for a calcium-mobilizing messenger at fertilization include cyclic ADP ribose[48] or cyclic GMP[108].

Once development begins, the embryo divides rapidly and each mitosis seems to be associated with an increase in calcium. In *Xenopus* embryos, the intracellular level of calcium oscillates during the cell cycle, reaching a peak at mitosis[109], when it seems to be responsible for triggering nuclear envelope breakdown at prophase[110]. Injection of $InsP_3$ can trigger breakdown of the nuclear envelope in sea urchin embryos[110]. A role for this messenger in controlling mitosis has been supported by experiments on *Xenopus* embryos in which injection of heparin or a monoclonal antibody against $PtdIns(4,5)P_2$ was found to arrest the cell cycle[111]. What remains to be determined, however, is how this messenger pathway interacts with maturation-promoting factor to orchestrate an orderly entry into mitosis.

As the embryo grows, the form of the final organism begins to emerge as each cell expresses its specific developmental programme including the establishment of polarity. Both $InsP_3$ and DAG have been implicated in setting up the dorso-ventral

BOX 3 Intracellular calcium channels



THE ryanodine receptor (RYR) is almost twice as large as the inositol trisphosphate receptor (IP_3R). The skeletal RYR1 (refs 31, 32) and cardiac RYR2 (ref. 33) display a 66% identity[33]. A much shorter RYR3 has been identified in some non-muscle cells[34]. The IP_3R_1 (refs 26, 27) is 70% homologous to IP_3R_2 (refs 28, 29). There are partial sequences for the C-terminal domains of two other receptors IP_3R_3 and IP_3R_4 (refs 28, 29). The primary sequences of members of the two families have regions of homology, particularly in their C-terminal domains which have the membrane-spanning regions. The inset shows the last two membrane-spanning regions and the C-terminal cytoplasmic tail of the skeletal RYR. Filled circles represent amino-acid identities with the IP_3R_{1a}. Dashed line represents the binding site of the monoclonal antibody 18A10, which can inhibit the calcium-mobilizing action of $InsP_3$ (ref. 35). Conversely, another antibody directed against this C-terminal region enhances the effects of $InsP_3$ (ref. 36). S1 and S2 splice sites: asterisks, cAMP- and calmodulin-dependent phosphorylation sites: arrows, cAMP-dependent phosphorylation sites; arrowhead, highly conserved TXCFICG motif in the free C-terminal domain.

Modulation of IP_3R activity

Various pharmacological agents can modulate the IP_3R but their actions are not particularly specific. For example, heparin can act as a competitive inhibitor but its usefulness for studies on intact cells is lessened because it may also inhibit the generation of $InsP_3$. The use of caffeine to activate RYRs is complicated by the observation that it is a potent inhibitor of the IP_3R (refs 37, 38). Caffeine abolishes the apparent cooperativity of $InsP_3$-induced calcium release, suggesting that it interferes with the event that couples the binding of $InsP_3$ to subsequent channel opening[39]. Finally, ethanol is a potent inhibitor of the IP_3R, which might account for the ataxia associated with ethanol intoxication[40].

axis in *Xenopus* embryos[112,113]. The level of $InsP_3$ suddenly increases 2-to 4-fold at the 32-64 cell stage which marks the onset of mesoderm induction when the embryo is most sensitive to the teratogenic effects of Li^+ (ref. 113). An inositol depletion hypothesis attempts to explain this effect of Li^+ on the basis that it inhibits inositol phosphate metabolism, so preventing the regeneration of free inositol[114]. The basic premise is that this depletion of inositol will desensitize phosphoinositide signalling by slowing down the resynthesis of the $PtdIns(4,5)P_2$ precursor used to release $InsP_3$. In keeping with this hypothesis, the spontaneous increase in $InsP_3$ at the time of mesoderm induction was completely suppressed by Li^+ treatment[113]. Furthermore, Li^+ treatment caused the expected fall in the level of inositol[113], and the injection of the latter was able to rescue embryos against the teratogenic effect of Li^+ (ref. 112). All this evidence strongly implicates a role for the phosphoinositides in early embryonic induction. Finally, it is of interest to note that expression of the *wnt-1* proto-oncogene can reproduce all the effects of Li^+, including the duplication of the dorsal axis[115], the increase in gap junctional communication between ventral cells[116], and the restoration of an axis when *wnt-1* was expressed on one side of an ultraviolet-irradiated embryo[115]. All these similarities suggest that, like Li^+, *wnt-1* might modulate the way cells respond to inducing agents by suppressing the level of phosphoinositide signalling.

In the case of mammals, there is indirect evidence to suggest a role for the phosphoinositides in early development. A genetic predisposition for neural tube defects in the curly tail (*ct/ct*) mouse can be overcome by culturing embryos in media containing inositol[117]. Also, there is a hint that the development of normal body asymmetry, namely left-right sidedness, may depend upon this signalling pathway. The *iv* (*situs inversus viscerum*) mutation in mice interferes with this left-right decision-making process so that half of the mutants display *situs inversus* (the normal asymmetry is reversed). This mutant phenotype is reproduced if rat embryos are cultured with the α_1-adrenergic agonist noradrenaline[118]. As the α_1-adrenergic receptor is invariably coupled to PLC (Box 1), $InsP_3$ or DAG are likely candidates to determine left-right handedness during embryonic development.

Cell growth

Within a fully developed multicellular organism, most cells are non-dividing (G0 phase of the cell cycle), but they retain the option of returning to the cell cycle usually when activated by growth factors. We are entering an exciting phase in which links are being forged between transduction events at the plasma membrane and the cell cycle proteins such as the cyclins and cyclin-dependent kinases which contribute to the DNA-synthesis and maturation-promoting factors which operate at G1/S and G2/M respectively. The role of calcium in stimulating maturation promoting factor at fertilization was described earlier and here I shall concentrate on the G1 events that culminate in activation of DNA synthesis-promoting factor. Other components of this factor are the tumour suppressors (like p53 and the retinoblastoma gene product) and transcription factors such as E2F and DRTF1. During the activation of the DNA synthesis-promoting factor, the inhibitory effect of the suppressors is removed once they become phosphorylated and leave the nucleus (Fig. 5).

The problem of cell cycle control can thus be re-phrased in more precise terms: how do growth factors acting at the cell surface bring about the onset of DNA synthesis by stimulating its promoting factor in the nucleus?

Cell cycle control is complicated by the profusion of growth factors acting on different messenger pathways (Figs 1 and 5). Both G protein-linked receptors (for example, bombesin, bradykinin, endothelin) and tyrosine kinase-linked receptors (for example, PDGF, EGF and insulin-like growth factor (IGF)) can stimulate proliferation. The phosphoinositide-derived signals $InsP_3$ and DAG are common to both pathways (Fig. 1), but there is conflicting evidence concerning their role in promoting cell proliferation. In certain cells an increase in phosphoinositide turnover is not in itself a sufficient stimulus to induce mitogenesis[119]. Chinese hamster lung fibroblasts transfected with M1 muscarinic receptors responded to carbachol with an increase in inositol phosphate release and *fos* and *myc* induction, but there was no DNA synthesis[119]. By contrast, the same M1 receptor was mitogenic in CHO cells[120]. The proliferation of glial cells during development of the mammalian brain may depend upon the stimulation of $PtdIns(4,5)P_2$ hydrolysis by such muscarinic receptors[120]. Proliferation of fibroblasts is inhibited by antibodies directed against $PtdIns(4,5)P_2$ (ref. 121) or PLC[122], whereas the injection of either PLC-β or PLC-γ stimulated DNA synthesis[123]. Furthermore, a toxin extracted from *Pasteurella multocida* is a very effective stimulus of the phosphoinositide system and is a potent mitogen[124].

Another way of trying to determine whether $InsP_3$ and DAG have a mitogenic function is to mimic their action with appropriate pharmacological agents. Lymphocytes can be activated using a combination of a phorbol ester or a permeant DAG analogue to activate PKC and an ionophore to mimic the calcium-mobilizing action of $InsP_3$. These agents must be present for prolonged periods, consistent with the observation that growth factors are required throughout G1. In hepatectomized rats, DNA synthesis begins at 14 h but can be inhibited by an α_1-adrenergic inhibitor applied as late as 11 h after partial hepatectomy[125]. Such inhibitors also prevent the increase in calmodulin within the nucleus of liver cells following hepatectomy[126]. Others have shown that calmodulin is required during G1 and may have a role in stimulating DNA synthesis-promoting factor[127]. If calcium influx is reduced during mitogenic stimulation either by lowering the level of external calcium[128] or by using a calcium channel inhibitor[129], there is a marked inhibition of cell growth.

Measurements of calcium in fertilized eggs[68,97] or lymphocytes[130] following mitogenic stimulation revealed the existence of repetitive calcium spikes which persisted for as long as several hours. As described earlier, $InsP_3$ plays a central role in driving this oscillatory activity and the wave-like spread of each spike may facilitate the transfer of a calcium signal to the nucleus. Indeed, there are indications that the calcium concentration within the nucleus increases rapidly after antigen stimulation of lymphocytes[131] or fertilization of sea urchin eggs (Fig. 3)[87]. In lymphocytes, antigen activates the $InsP_3$/calcium signalling pathway (Fig. 1) that contributes to gene transcription early in G1 (Fig. 5). One of the actions of calcium is to stimulate the translocation of transcriptional factors (NF-AT and NF-IL2A for example) from the cytoplasm into the nucleus. Calcium stimulates the calmodulin-dependent protein phosphatase calcineurin, which is one of the targets for the immunosuppressant drugs cyclosporin A (Fig. 5) and FK-506 (ref. 132). Overexpression of calcineurin makes T cells more resistant to cyclosporin A and FK-506 (ref. 133). Furthermore, this enhanced presence of calcineurin can act in synergy with a phorbol ester to stimulate T cells, thus by-passing the normal calcium requirement[133].

All this evidence suggests that the $InsP_3$/calcium signalling pathway is activated by mitogenic stimuli and may contribute to the changes culminating in DNA synthesis. Activation of transcription factors which then translocate into the nucleus indicates that some of the actions of calcium occur in the cytoplasm. But there is also evidence that the level of nuclear calcium increases dramatically following mitogenic stimulation[87,131], suggesting that calcium may also act within the nucleus. One of the functions of calcium is to activate MAP II kinase[134], which is part of the phosphorylation cascade that culminates in the activation of DNA synthesis-promoting factor (Fig. 5).

Autonomous nuclear signalling

The nucleus contains an autonomous phosphoinositide signalling system every bit as complex as that present at the plasma membrane[135,136]. It contains the lipid kinases necessary to phosphorylate PtdIns to both PtdIns(4)P and PtdIns(4,5)P_2, which are present both in the nuclear membrane and within the nuclear matrix, which also contains PLC-β[137]. What is particularly interesting is that this nuclear PLC-β is activated when Swiss 3T3 cells are stimulated by IGF-1, whereas bombesin has no effect even though it is a potent activator of phosphoinositide hydrolysis at the plasma membrane[135,137]. As a result of this hydrolysis, $InsP_3$ and DAG will be formed in the nucleus where they might perform their familiar second messenger functions. Isolated nuclei display an ATP-dependent uptake of calcium, of which ~20% was subsequently released by $InsP_3$ (ref. 138). The most likely explanation is that calcium stored within the space between the nuclear membranes was released to the cytosol when $InsP_3$ bound to receptors on the outer nuclear membrane. But such a mechanism would not explain the increase in nuclear calcium that occurs following activation of lymphocytes[131] or sea urchin eggs[87]. At present there is no evidence for IP_3Rs on the inner nuclear membrane, so the function of any $InsP_3$ that might be generated in the nucleus remains a problem.

Cell transformation

Cancer cells become tumorigenic as a result of multiple independent steps which subvert the normal growth control mechanisms described earlier. Some of these steps have been linked with mutations that either activate proto-oncogenes such as *ras* and *myc*, or remove the inhibitory action of tumour-suppressor genes such as *Rb* and *p53* (Fig. 5). Such tumorigenesis has been induced in cultured cells following transfection with some of the 7-membrane-spanning receptors that are coupled to PLC (Fig. 1 and Box 1) such as 5-HT_{1c} (ref. 139), M1, M2 and M5[140], and α_{1B}-receptors[16]. Similarly, expression of a mutated α_q subunit, which enhanced basal PLC activity, was found to transform NIH 3T3 cells but not Rat-1 cells[141]. These receptors and G proteins may be considered as new classes of proto-oncogenes that might transform cells by enhancing the phosphoinositide-derived signals $InsP_3$ and DAG. For example, the phorbol ester TPA (12-*O*-tetradecanoylphorbol 13-acetate), which mimics the action of DAG, can co-operate with the oncogene *ras* to transform primary rat embryo fibroblasts[142]. With regard to calcium, cell transformation leads to a much reduced calcium requirement for growth[143].

Neuromodulation and synaptic plasticity

The brain is the richest source of the components of the phosphoinositide signalling pathway. For example, cerebellar Purkinje cells have a very high density of IP_3Rs and have thus featured significantly in the purification[62], cloning[26] and fine-structural localization[41,46] of this receptor. The brain has also been important for studying the organization of the PKC family[8] which is colocalized with the IP_3R system. The IP_3R is particularly plentiful in the cerebellum, hippocampus, cerebral cortex, corpus striatum and olfactory tubercule[144]. It is unlikely that the $InsP_3$/calcium pathway has any role to play in the rapid signal transfer mediated by ionotropic receptors, but there is growing evidence that many metabotropic receptors employ phosphoinositide-derived signals to modulate both neural activity and the neural plasticity responsible for memory[5]. The recent report that presynaptic mGlu receptors employ phosphoinositide-derived signals as a positive feedback mechanism to enhance glutamate release is particularly interesting[145].

We still do not know why the large cerebellar Purkinje neurons and CA1 hippocampal neurons have such large numbers of IP_3Rs. The *staggerer* mutant mouse has a genetic lesion that prevents the expression of IP_3Rs specifically in the Purkinje neurons, which are poorly developed[146]. Perhaps IP_3Rs are required for the development of the extensive arborization of the dendritic tree. Another possibility is that the IP_3R functions to integrate information from the multiple inputs received by this dendritic tree. The co-existence of IP_3Rs and RYRs may somehow help to integrate information coming in from the outside and to relay it throughout the neuron through the process of CICR already described[64]. Indeed, there is a striking increase in nuclear calcium when neurons are depolarized[147].

Purkinje and hippocampal neurons are noted for their plasticity because they show long-term changes in neural transmission following certain stimulus regimes. Paired activation of the parallel and climbing fibre inputs results in long-term depression of the parallel fibre-Purkinje cell synapse, which is thought to be mediated through the mGlu receptor that is coupled to $InsP_3$ formation (Box 1)[148]. The synaptic spines on the Purkinje cells, which receive the input from the parallel fibres, contains an ER system rich in IP_3Rs[46]. Within the hippocampus, long-term potentiation (LTP) of synaptic transmission can be induced by the large calcium signal resulting from a brief tetanic stimulation of NMDA (*N*-methyl-D-aspartate) receptors. The phosphoinositide signalling pathway may have a role to play because this tetanus-induced onset of LTP was enhanced by activation of the mGlu receptor[149]. Indeed, activation of the mGlu receptor alone can bring about LTP in both the hippocampus[150] and in the dorsolateral septal nucleus[151]. As this mGlu receptor is coupled through a pertussis toxin-sensitive G protein (see Box 1), it is significant that LTP is suppressed by pretreatment with pertussis toxin[152]. The hippocampus receives a large cholinergic innervation which may also play a role because LTP can be enhanced by the cholinesterase inhibitor physostigmine but is reduced by the anticholinergic agent scopolamine[152]. The memory loss we all begin to experience with age may result from a similar impairment of the cholinergic system, which may also be altered in Alzheimer's disease.

This apparent role of the cholinergic system in enhancing synaptic activity seems to be mediated by the phosphoinositide

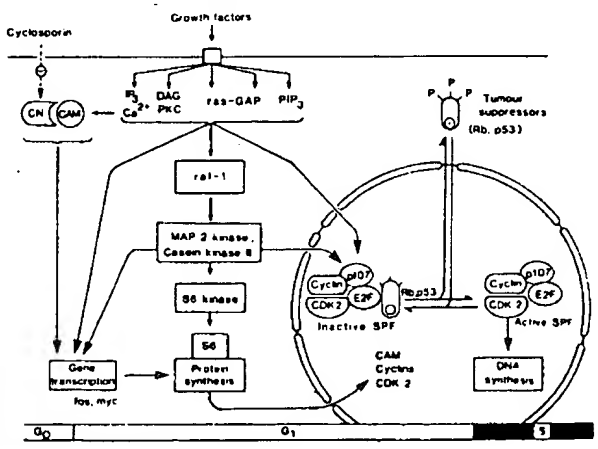


FIG. 5 Summary of signalling pathways operating during G_1. Growth factors generate a number of putative mitogenic messengers which then feed into various pathways to control both early events at the G0/G1 boundary (for example, *fos* and *myc* transcription) and later events which culminate in the onset of DNA synthesis. One important signalling cascade involves *ras*, raf 1 and MAP2 kinase, which can then activate both S6 kinase and DNA synthesis-promoting factor (SPF). SPF contains cyclin, p107, the transcription factor E2F and tumour suppressors such as the retinoblastoma gene product (Rb) and p53. GAP, GTPase-activating protein; CAM, calmodulin; CN, calcineurin; DAG, diacylglycerol; PKC, protein kinase C.

system. The marked potentiation of NMDA responses by acetylcholine in CA1 neurons was reproduced by the flash photolysis of caged $InsP_3$ (ref. 153) but was blocked by the IP_3R antagonist heparin[153]. Furthermore, this potentiation was lost in slices prepared from Li^+-treated rats but could be restored following addition of free inositol, which is entirely consistent with the inositol depletion hypothesis[114]. Most attention has been focused on the ionotropic receptors (like NMDA and kainic acid receptors), but it is clear that metabotropic receptors operating through the $InsP_3$ and DAG messenger systems have a profound effect on neural behaviour, including memory.

Sensory perception

The phosphoinositide signalling pathway has been adapted for detecting external cues by invertebrate photoreceptors[154], olfactory sense organs in insects[155], lobsters[36] and fish[77], and in certain mammalian taste cells[156]. As for hormonal pathways, these sensory transducing mechanisms use conventional G protein-linked receptors to generate $InsP_3$, for example the rhodopsin-$DG_{\alpha q}$-norpA pathway (Box 1) in *Drosophila* photoreceptors. The ER cisternae lying immediately below the photoreceptive microvillar membrane respond to $InsP_3$ by releasing calcium, which then opens sodium channels in the surface membrane to give the receptor potential. During prolonged illumination of *Drosophila* photoreceptors, this receptor potential is maintained but decays in transient receptor potential (*trp*) mutants in which the calcium influx component necessary for a maintained response is missing[154]. The *trp* gene encodes a plasma membrane channel with close homology to the dihydropyridine receptor[157]. Minke and Selinger[154] have speculated that the *trp* gene codes for a plasma membrane channel that interacts with an IP_3R to gate calcium through the capacitative mechanism described earlier.

Olfactory and taste organs closely resemble conventional hormonal systems in that they respond to chemical signals by membrane-bound receptors coupled to various second messengers. A role for cyclic AMP in olfactory and taste organs is well-established, but $InsP_3$ has also been implicated for detecting certain chemical modalities[158]. Our ability to enjoy sweet substances depends upon the activation of adenylyl cyclase, whereas bitter tastes are detected through $InsP_3$ (refs 77, 159). A subpopulation of taste cells in the *circumvallate papillae* have IP_3Rs highly concentrated near the pore where most of the taste receptors are located[156]. Rapid increases in $InsP_3$ levels occur in olfactory epithelia in response to pyrazine[159] and in taste cells responding to the intensely bitter substance denatonium[156]. Both electrophysiological[77] and biochemical[76] evidence suggests that $InsP_3$ acts on a calcium channel of M_r 107K which is located in the plasma membrane of catfish olfactory cells.

The temporal responsiveness of insect olfactory organs is truly remarkable. Within 50 ms of applying a specific sex pheromone, the $InsP_3$ level in cockroach antennae increases from 60 to 2,540 pmol mg^{-1} protein[155]. This rapid production of $InsP_3$ is fast enough to account for the odorant-induced generator potentials which appear after a latency of 100–200 ms. All this evidence indicates a central role for $InsP_3$ in certain forms of sensory perception. It has been suggested that the cyclic AMP and $InsP_3$ messenger pathways may interact with each other to enhance odorant discrimination[158].

Conclusion

Over the past decade there has been enormous progress in our understanding of how cells use calcium to regulate their activity. The second messenger $InsP_3$ has a key role in controlling both the mobilization of internal stores and the entry of external calcium. Cells generate $InsP_3$ through two major signalling pathways. In one pathway, receptors are coupled through G proteins to stimulate PLC-β, whereas the other pathway depends upon tyrosine kinase-linked receptors which are specifically coupled to PLC-γ. When activated, these receptors undergo autophosphorylation on specific tyrosine residues, providing docking sites that bind to PLC-γ and result in its activation.

Once $InsP_3$ is released to the cytosol it mobilizes calcium by binding to receptors. Molecular studies have revealed a family of IP_3Rs that have many structural and physiological similarities to RYRs, another major family of intracellular calcium channels. In particular, both families display an autocatalytic process of calcium-induced calcium release which is responsible for both the temporal and spatial patterns of calcium signalling. When cells are stimulated they invariably display regular calcium spikes which often appear as waves. The IP_3R contributes to these complex patterns in two ways. It controls the influx of calcium, which maintains this activity, and it is responsible for the regenerative release of calcium during each spike.

Now that the major aspects of the $InsP_3$-calcium signalling pathway have been mapped out, attention is beginning to focus on its role in specific cellular responses. This pathway is fully operational in germ cells, where it has been implicated in both fertilization and early development. There is continuing support for the hypothesis that Li^+ re-specifies the dorso-ventral axis in amphibians by repressing the spontaneous surge of $InsP_3$ in the early blastula. Once cells differentiate, the $InsP_3$-calcium pathway is adapted to control many responses such as smooth muscle contraction, liver metabolism and stimulus-response coupling in many secretory cells. Many differentiated cells retain the ability to return to the cell cycle when confronted with the appropriate mitogenic stimulus. Many growth factors are capable of stimulating the phosphoinositide signalling pathway but there are conflicting opinions as to its role in regulating cell growth. It seems that the $InsP_3$-calcium and DAG-PKC pathways can contribute to the sequence of events that culminates in DNA synthesis, particularly in primary cells such as lymphocytes, liver and glial cells. In addition, there are also indications that this signalling pathway can induce cells to become tumorigenic.

The nervous system is a rich source of all the components of the phosphoinositide signalling pathway but the function of $InsP_3$ and DAG has been difficult to establish. There is evidence that $InsP_3$ functions in sensory perception (taste, olfaction and invertebrate photoreception). Perhaps the greatest challenge for the future is to understand how this signalling pathway operates in the CNS. There is already substantial evidence for a role in modulating synaptic plasticity and we can anticipate that both $InsP_3$ and DAG will regulate many other neuronal processes. □

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Inhibitors of Type IV Phosphodiesterases Reduce the Toxicity of MPTP in Substantia Nigra Neurons *In Vivo*

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Abstract

The neuropathology of Parkinson's disease is characterized by the degeneration of dopaminergic neurons in the substantia nigra. We have recently shown that the activation of protein kinase A improves the survival of dopaminergic neurons in culture and, furthermore, protects them from the dopaminergic neurotoxin, 1-methyl-4-phenylpyridinium ion (MPP^+) *in vitro*. We have now analysed the potential of phosphodiesterase inhibitors to increase cAMP levels in dopaminergic neurons, to improve their survival in culture and to protect them from the toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) *in vivo*. Increasing intracellular cAMP with phosphodiesterase type IV-specific inhibitors enhanced the survival of dopaminergic neurons in culture. Inhibitors of other phosphodiesterase types were not active. *In vivo*, phosphodiesterase type IV inhibitors reduced the MPTP-induced dopamine depletion in the striatum of C57BL/6 mice. Furthermore, the loss of tyrosine hydroxylase-immunopositive neurons in the substantia nigra of these animals was diminished. After Nissl staining, a similar reduction of the MPTP-induced loss of neurons was observed in the substantia nigra. The protective effect of protein kinase A activation did not appear to be due to the blocking of MPP^+ uptake into dopaminergic neurons. This was not decreased after treatment with forskolin or 8-(4-chlorophenylthio)-cAMP. Thus, protein kinase A regulates the survival and differentiation of dopaminergic substantia nigra neurons *in vivo*, implicating a therapeutic potential for substances which regulate cAMP turnover in these neurons.

Introduction

Mesencephalic dopaminergic neurons have raised considerable interest because of their involvement in pathological conditions such as Parkinson's disease and schizophrenia. Currently, no treatment is available which prevents or significantly retards the progressive degeneration of this neuron group in Parkinson's disease. In an analysis of the influence of synaptic input on the survival of mesencephalic dopaminergic neurons we have recently demonstrated that their *in vitro* survival was increased by exposure to dibutyryl-cAMP or forskolin during the first 3 days after plating (Hartikka *et al.*, 1992). This effect could be blocked by inhibitors of cAMP-dependent protein kinases. Neither phorbol esters nor factors like insulin-like growth factor I, basic fibroblast growth factor (bFGF) or factor released from activated astrocytes improved the survival of these neurons under the same culture conditions, although the factors stimulated dopamine uptake (Gaul and Lübbert, 1992; Hartikka *et al.*, 1992). Other authors have subsequently described a similar action of cAMP on central noradrenergic neurons (Sklair-Tavron and Segal, 1993). In addition, phosphorylation by protein kinase A may have long-lasting effects on the transmitter release of dopaminergic and serotonergic neurons since it regulates the expression of genes involved in the electrical activity of the neurons (Roguet *et al.*, 1993).

We also showed that, under the culture conditions used, cAMP-dependent protein kinases, but neither bFGF nor a trophic activity released from activated astrocytes, protected dopaminergic neurons from degeneration induced by the 1-methyl-4-phenylpyridinium ion (MPP^+) (Gaul and Lübbert, 1992; Hartikka *et al.*, 1992). The dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is converted to MPP^+ by monoamine oxidase B, incorporated into dopaminergic neurons via the dopamine uptake system and then actively transported into mitochondria, where it decreases the complex I activity of the respiratory chain, increases the calcium permeability of the mitochondrial membrane and produces free radicals (Sundstrom *et al.*, 1990; Heikkila and Sonsalla, 1992; Ramsay and Singer, 1992; Tipton and Singer, 1993). Cell death follows probably via apoptosis, since MPP^+ has been shown to induce apoptosis in both cerebellar neurons (Dipasquale *et al.*, 1991) and mesencephalic–striatal cocultures containing the highly sensitive dopaminergic neurons (Mochizuki *et al.*, 1994). The late stages of the MPTP model for Parkinson's disease show interesting parallels to the pathological process in the disease (Schapira *et al.*, 1990; Fahn and Cohen, 1992).

To build a realistic pharmacological strategy based on these findings

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we have now analysed the potential of phosphodiesterase (PDE) inhibitors to improve the survival of dopaminergic neurons *in vitro* and to protect C57BL/6 mice from the toxicity of MPTP. We found that inhibitors of type IV PDE, but not of other PDE types, substantially reduce MPTP toxicity *in vitro* and *in vivo*.

Materials and methods

Enzymatic activity of partially purified phosphodiesterases

Phosphodiesterase activity was assayed by a batch method as described (Thompson *et al.*, 1979). Cells and tissues were homogenized on ice in 10 mM Tris–HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM dithiothreitol, 1 μg/ml leupeptin and 0.17 mg/ml phenylmethylsulphonyl fluoride (PMSF; added just before homogenization). Neutrophils (for PDE type IV) and platelets (for PDE types II and III) were obtained from human blood and sonicated (Branson probe, 4 × 15 s). Human lung was from patients undergoing surgery and was homogenized (Polytron homogenizer, two bursts of 30 s). PDE type III and IV (substrate 1 μM cAMP) preparations consisted of low-speed supernatants of the platelet and neutrophil homogenates, respectively. Types I, II and V were separated by anion exchange chromatography (Q-Sepharose) using a gradient of NaCl in homogenization buffer without sucrose and PMSF (0–0.1 mM NaCl in 2.5 column volumes, 0.1–0.45 mM in 24 column volumes).

PDE I. Fractions where hydrolysis of 1 μM cAMP could be stimulated by Ca^{2+}/calmodulin (0.5 mM and 125 nM, respectively) eluted at 0.17–0.18 M NaCl.

PDE II. Fractions showing substantial cAMP hydrolytic activity at 100 μM but not at 1 μM eluted at 0.31–0.32 M NaCl.

PDE V. Fractions selectively hydrolysing 1 μM cGMP over 1 μM cAMP eluted at 0.2–0.24 M NaCl.

Cell culture, dopamine and MPP^+ uptake and survival of dopaminergic neurons

Primary cultures containing dopaminergic neurons were prepared from embryonic day 14 fetal rat ventral mesencephalon as described (Hartikka *et al.*, 1992). A 15 mm culture well usually contained 6 × 10^5 neurons. Dopamine and MPP^+ uptake was measured as described (Hartikka *et al.*, 1992) using tritiated dopamine or MPP^+ at concentrations of 50 nM (specific activity 45 Ci/mmol, New England Nuclear) or 1 μM (specific activity 80 Ci/mmol, New England Nuclear), respectively. The survival of dopaminergic neurons in the culture was assayed by counting tyrosine hydroxylase-positive neurons previously stained with a mouse anti-tyrosine hydroxylase antibody (Boehringer Mannheim) (Hartikka *et al.*, 1992).

MPTP-injected mice

Eight-week-old male C57BL/6 mice were subcutaneously injected with MPTP (10 or 20 mg/kg as indicated in the figure legends) or saline. A second and in some experiments third injection of MPTP followed 2 and 4 h later. One hour before the first MPTP injection, between the injections and 1 h after the last MPTP injection, the mice were subcutaneously injected with a PDE inhibitor dissolved in 1 ml of polyethylene glycol-400 and then diluted with 9 ml saline. Controls were injected with 10% polyethylene glycol in saline. Non-toxic doses for each substance were determined *in vivo* in trial experiments. At higher concentrations, there was increased lethality in mice which received a combination of inhibitor and MPTP. Seven days after the injections the animals were killed and the monoamines were determined by HPLC as described (Schneider *et al.*, 1992). Alternatively, the mice were intracardially perfused with 4% paraformaldehyde, and the whole brain was further fixed in cold 4% paraformaldehyde overnight and processed for tyrosine hydroxylase immunocytochemistry or Nissl-stained with toluidine blue. Cryosections (40 μm) were cut in the midbrain region, blocked for endogenous peroxidases with 0.3% peroxide in methanol for 15 min and digested with 0.1% trypsin in phosphate-buffered saline (PBS) for 10 min. This was followed by a 48-h incubation at 4°C with a polyclonal rabbit antiserum against tryosine hydroxylase (Affinity Research Products Ltd, Exeter, UK) diluted 1:100 in PBS with 2% goat serum and 0.2% Triton-X. Sections were further developed using the Vectastain Elite ABC Kit (Vector Laboratories) and nickel–diaminobenzidine (DAB) enhancement. Prior to the DAB reaction, sections were incubated for 10 min in 2% $NiSO_4$ in 0.1 M Tris–HCl, pH 7.4. The solution was removed and replaced with 0.05% DAB,

TABLE 1. Isoenzyme specificities of phosphodiesterase inhibitors on partially purified human enzymes

Isoenzyme	Type I	Type II	Type III	Type IV	Type V
MIMX	4.83 ± 0.18	3.84 ± 0.05	3.37 ± 0.03	3.66 ± 0.09	4.83 ± 0.08
Zaprinast	4.82 ± 0.10	<3.5	<3	3.54 ± 0.16	5.40 ± 0.13
Milrinone	3.52 ± 0.23	3.23 ± 0.35	5.52 ± 0.01	4.31 ± 0.05	3.90 ± 0.09
Amrinone	<3	<3	4.38 ± 0.16	<3	3.18 ± 0.35
IBMX	4.57 ± 0.05	4.19 ± 0.13	4.98 ± 0.10	4.71 ± 0.05	4.73 ± 0.05
XT-A	3.47 ± 0.24	3.68 ± 0.12	3.70 ± 0.06	5.92 ± 0.19	3.80 ± 3.31
NQ-A*	<4.5	<4.5	<4.5	5.10 ± 0.08	n.d.
Ro-201724	<3	<3	3.47 ± 0.16	5.42 ± 0.27	3.09 ± 0.31
SDZ-MNS 949	3.93 ± 0.06	3.63 ± 0.08	5.37 ± 0.10	6.32 ± 0.01	4.43 ± 0.12

Negative logarithms of IC_{50} values (pIC_{50}) are listed with 95% confidence intervals. Semipurified human enzymes were prepared and PDE activity determined as described in Materials and methods. Inhibitory potency was calculated by fitting concentration–inhibition curves encompassing the IC_{50} to the two-parameter logistic equation (seven data points). Inhibitors: MIMX is 8-methyoxymethyl-3-isobutyl-1-methylxanthine (Beavo and Reifsnyder, 1990), zaprinast and milrinone are described in Beavo and Reifsnyder (1990), amrinone in Harrison *et al.* (1986), IBMX is 3-isobutyl-1-methylxanthine (Beavo and Reifsnyder, 1990), XT-A the xanthine analogue 1,3-dicyclopropylmethyl-8-aminoxanthine (Esser, 1992), NQ-A the nitroquazone analogue 1-(3-carbomethoxyphenyl)-3-benzyl-quinazoline-2,4-dione (Lowe *et al.*, 1991), Ro-201724 is described in Beavo and Reifsnyder (1990), and SDZ-MNS 949 (MNS) in Laengle *et al.* (1993/94). Except for IBMX (Sigma), the inhibitors were synthesized at Sandoz Pharma Ltd, Basel. Chemical formulae and synthesis information for all compounds are given in the references.

*Precipitation occurs above 30 μM.

n.d., not determined.

0.2% $NiSO_4$, 0.3% H_2O_2 in 0.1 M Tris. Nissl staining was carried out on alternate perfused sections using a 0.5% solution of toluidine blue in acetate buffer for 1 min.

Cell counting

Cells stained for immunoreactivity in the substantia nigra compacta (SNC) and the ventral tegmental area (VTA) were counted in every second coronal 40 µm section between levels 46 and 54 in the atlas by Paxinos and Watson (1982) taken from five animals in each treatment group (n = 60). Alternate sections were stained with toluidine blue and neurons in the SNC region were counted automatically using an MCID image analysis system (Imaging Research). Staining and analysis using the two methods were carried out by independent researchers.

Results

Specificity of phosphodiesterase inhibitors

Seven types of phosphodiesterases can be distinguished based on their biochemical properties (Beavo and Reifsnyder, 1990). Type I

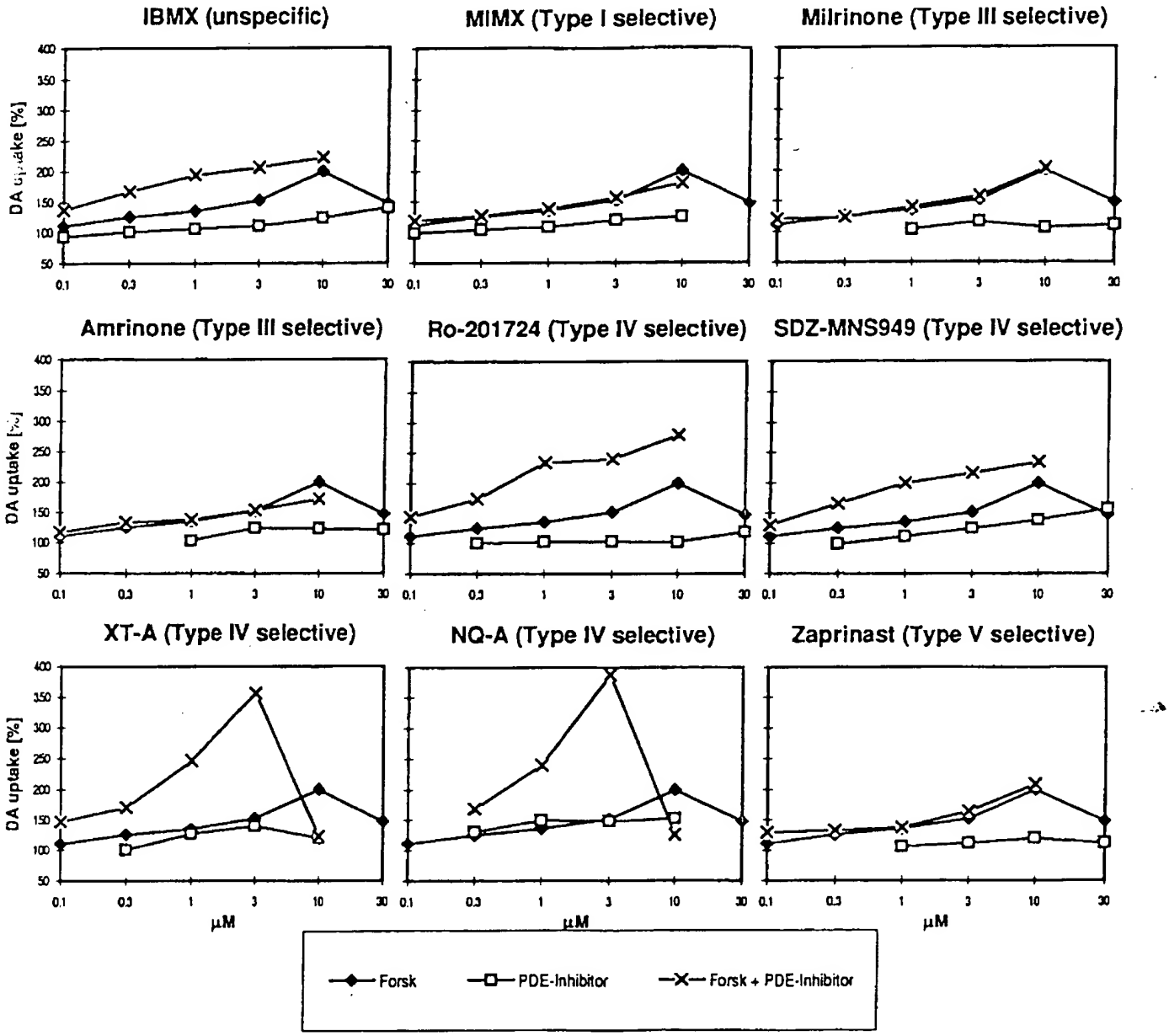


FIG. 1. Prestimulation with PDE type IV inhibitors but not PDE I, III and V inhibitors increases dopamine uptake in primary cultures treated with forskolin. Mesencephalic cultures were incubated for 3 days with increasing concentrations of forskolin or PDE inhibitors. In parallel experiments, the same increasing concentrations of forskolin were combined with 0.3 µM of the inhibitors except IBMX, which was used at 10 µM. After the 3 days, dopamine uptake was assayed as a measure of dopaminergic neuron survival and differentiation. Results are displayed as percentages of [^{3}H]dopamine uptake of untreated control cultures. Each graph in the figure presents the dose–response curves for the indicated inhibitor, forskolin as a basis for comparison, and forskolin combined with that inhibitor. The subtype selectivity of the PDE inhibitors is indicated (compare Table 1). Shown are the means of three independent experiments, each with four wells per treatment group. The apparent toxicity at higher concentrations of forskolin, particularly in combination with XT-A or NQ-A, was not observed in every experiment (compare the representative experiment shown in Fig. 2A). Standard errors were <10% for each data point.

PDE is Ca^{2+}/calmodulin-dependent, type II is stimulated and type III inhibited by cGMP, and types IV and V are specific for cAMP and cGMP, respectively (Beavo and Reifsnyder, 1990). Some additional PDE types were recently discovered and are less well characterized: type VI is a photoreceptor PDE, type VII PDE is highly cAMP-specific but rolipram-insensitive (Bolger *et al.*, 1993; Michaeli *et al.*, 1993), and type VIII is a rolipram-insensitive PDE with similar K_m values for cAMP and cGMP hydrolysis (Mukai *et al.*, 1994). We have determined the specificities of several phosphodiesterase inhibitors on partially purified PDEs of types I, III, IV and V (Table 1) and found several relatively specific inhibitors.

Type IV-specific PDE inhibitors potentiate the forskolin-induced increase in dopamine uptake and survival of dopaminergic neurons

Increasing concentrations of forskolin or PDE inhibitors were added to cultures of mesencephalic neurons immediately after plating (Fig. 1). After a 3-day exposure, dopamine uptake in the cultures was measured as described before (Hartikka *et al.*, 1992). The uptake was moderately increased by PDE inhibitors with high affinity for the cAMP-specific type IV phosphodiesterase. To simulate the situation of an *in vivo* treatment, a constant subcritical concentration of inhibitors (0.3 μM for all inhibitors except 3-isobutyl-1-methylxanthine (IBMX), which was used at 10 μM) was then applied together with increasing concentrations of forskolin, which should mimic synaptic input, activating adenylate cyclase. Some inhibitors greatly potentiated the forskolin-induced increase of dopamine uptake (Fig. 1). In several although not all experiments, forskolin and the inhibitors became toxic for the cultures above a certain concentration (compare Figs 1 and 2A). This difference may be due to the natural variation inherent in primary neuron cultures. The toxicity cannot, however, simply be related to the increase in cAMP since it happened at levels of stimulation which vary for different compounds, implying that the toxicity is partly or fully related to other actions of these molecules. Similar results were obtained in experiments where increasing concentrations of inhibitors were added in the presence of 0.1 μM forskolin (data not shown). In general, the potencies of the inhibitors in this assay reflected their affinities for the type IV PDEs (Table 1). Clearly, specific inhibitors for the type I, III, or V PDEs were not active. Participation of the cGMP-stimulated PDE type II can also be excluded based on the lack of an effect of cGMP (Hartikka *et al.*, 1992). PDE types VI, VII and VIII are also not relevant for this study since type VI PDE is restricted to photosensitive cells while types VII and VIII are insensitive to Ro-201724, a compound that was active in our experiments. The stimulation of dopamine uptake elicited by the PDE type IV inhibitors indeed reflected an improved survival of tyrosine hydroxylase-positive neurons in the culture, as shown in Figure 2A. The number of dopaminergic neurons after a 3 day treatment with 0.1–10 μM of forskolin and 0.3 μM of a nitraquazone analogue (NQ-A) was increased ~3-fold compared to the untreated controls. In addition, a 24 h pretreatment of older 5-day cultures with NQ-A and a prestimulating dose of 8-(4-chlorophenylthio)-cAMP (cpt-cAMP) protected the dopaminergic neurons from the toxicity of MPP^+ (Fig. 2B), indicating a direct protective effect of PDE IV inhibitors against the active metabolite of MPTP.

Treatment with type IV PDE inhibitors reduces the MPTP-induced dopamine depletion and the degeneration of dopaminergic substantia nigra neurons in vivo

Type IV-specific PDE inhibitors were then tested for their ability to protect dopaminergic substantia nigra neurons *in vivo*. Adult

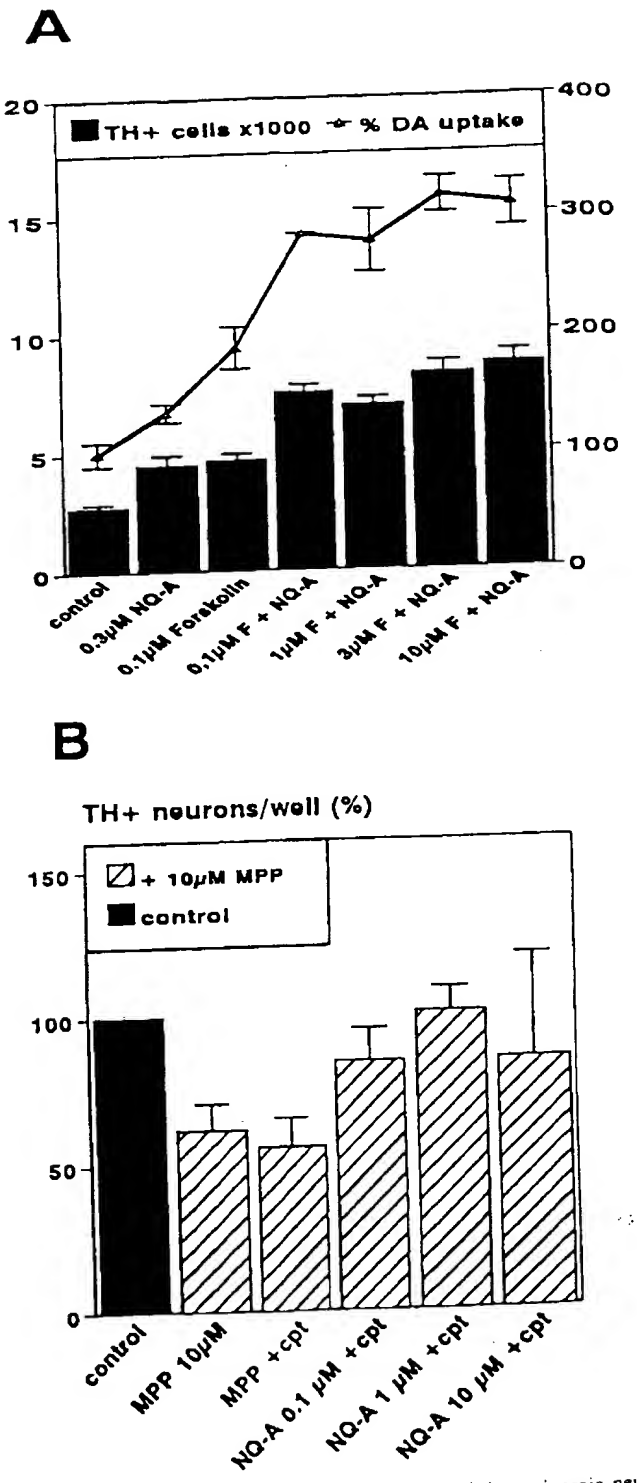
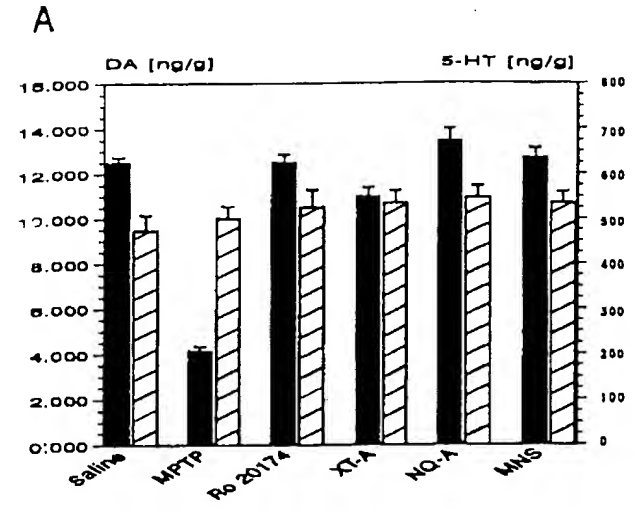
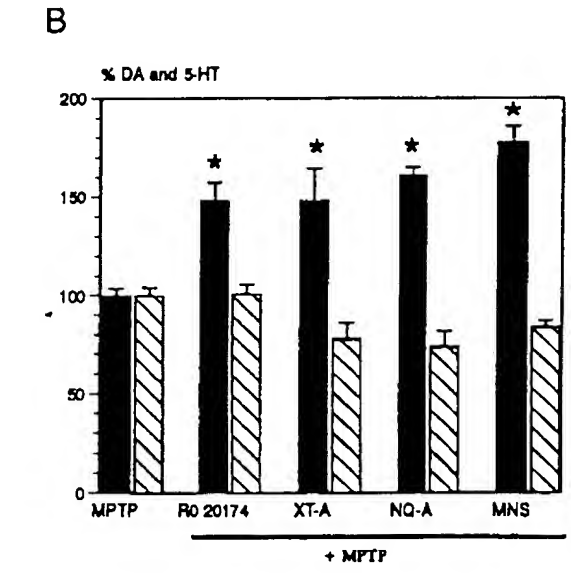
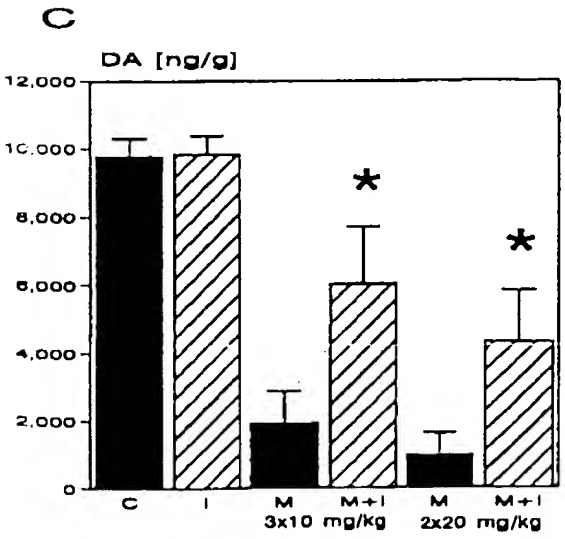


FIG. 2. (A) Correlation between dopamine uptake and dopaminergic neuron survival in primary cultures of fetal rat ventral mesencephalon after a 3 day treatment with increasing amounts of forskolin (F) and prestimulation with 0.3 μM of the PDE IV inhibitor NQ-A. Values are given ± SE, $n = 4$. (B) Protection of dopaminergic neurons in culture from the neurotoxin MPP^+ by a PDE IV inhibitor. As indicated in the figure, 5-day-old cultures were treated with 50 μM cpt-cAMP and increasing amounts of the PDE inhibitor NQ-A. One day later, 10 μM MPP^+ was applied and 48 h later the cells were stained with a tyrosine hydroxylase antibody and counted. Values from four independent experiments, each with four culture wells per data point, were converted to percentages for ease of comparison. Cell numbers in untreated control cultures were assigned 100% value in each experiment (therefore they have no variation). Values are given ± SEM, $n = 4$.







C57BL/6 mice received three subcutaneous injections of inhibitor interspersed with two injections of 10 mg/kg MPTP (Fig. 3). Seven days later, the dopamine content was assayed in the striatum, the area to which dopaminergic substantia nigra neurons project. This dose of MPTP caused a reduction of striatal dopamine to ~30% of control values (Fig. 3A). Treatment with PDE inhibitor alone did not significantly change the striatal dopamine content. Coinjection of MPTP with type IV PDE inhibitors from various chemical classes decreased the MPTP-induced depletion of striatal dopamine. In striata from mice injected with MPTP plus PDE IV inhibitor, the dopamine content was up to 80% higher than in animals treated with MPTP alone (Fig. 3B). Pronounced protective effects were also obtained when higher doses of MPTP were used (Fig. 3C). Three doses of 10 mg/kg MPTP reduced striatal dopamine to 20% of control values. Simultaneous treatment with PDE IV inhibitor reduced this loss to ~60% of controls. An even more severe lesion was produced with two injections of 20 mg/kg MPTP, striatal dopamine content being reduced to 10% of control values. Coinjection with the inhibitor maintained dopamine levels at ~44%.

In addition to the reduced dopamine decline in the striatum, we found a profound reduction in the loss of dopaminergic neurons in the substantia nigra. After injection of high doses of MPTP (3 × 20 mg/kg at 2 h intervals) the number of tyrosine hydroxylase-immunopositive neurons was reduced by 37% in the ventral tegmental area and paranigral nucleus and by 76% in the substantia nigra compacta (Fig. 4A). No changes in cell number or fibre density were apparent after injection of a PDE IV inhibitor without MPTP. When MPTP treatment was combined with a type IV PDE inhibitor, the cell number was at control levels in the VTA and paranigral nucleus, and at 60% of the control levels in the substantia nigra (Figs 4A and 5A–F). Furthermore, the density of tyrosine hydroxylase-positive fibres was considerably increased in mice injected with MPTP and the inhibitor compared to mice which were treated with MPTP alone (Fig. 5A–F). Since tyrosine hydroxylase expression can be stimulated by cAMP, we also counted the number of neurons in the SNC after Nissl-staining alternate sections with toluidine blue (Figs 4B and 5G–I). Tyrosine hydroxylase analysis was performed manually, while Nissl-stained sections were analysed automatically by a different investigator using an MCID imaging system. The results paralleled those described for the counting of tyrosine hydroxylase-positive cells (Fig. 4B).

FIG. 3. Type IV PDE inhibitors decrease the MPTP-induced dopamine depletion in the striatum of C57BL/6 mice. (A) Animals were injected three times at 2 h intervals with the indicated PDE inhibitors [10 mg/kg Ro-20174, 1 mg/kg XT-A or NQ-A, or 30 mg/kg of the isoquinoline SDZ-MNS 949 (MNS)] without MPTP injections. Depicted are the amounts of dopamine (solid bars) and serotonin (hatched bars) in the striatum 7 days later. (B) Animals received two MPTP injections (10 mg/kg) interspersed between the three PDE inhibitor injections. Dopamine and serotonin levels are expressed as percentages of those found in MPTP-injected mice. Values from at least three independent experiments (each with $n > 4 \pm$ SE) are shown. The protection from MPTP-induced dopamine depletion by the PDE IV inhibitors was statistically significant according to the *t*-test (*$P < 0.01$, two-tailed distribution, two-sample unequal variance). (C) Higher doses of MPTP were used to induce a more severe lesion. Animals injected three times with 10 mg/kg MPTP (total 30 mg) received four interspersed injections of PDE IV inhibitor, while animals injected twice with 20 mg/kg MPTP (total 40 mg) received three injections of PDE IV inhibitor. Hatched bars indicate treatment with multiple doses of 10 mg/kg isoquinoline. The protection from MPTP-induced dopamine depletion by the PDE IV inhibitor was statistically significant according to the *t*-test (*$P < 0.001$). Treatment groups consisted of six or more animals in each case. C, vehicle control; M, MPTP-injected; M+I, injected with MPTP plus inhibitor; I, inhibitor alone.

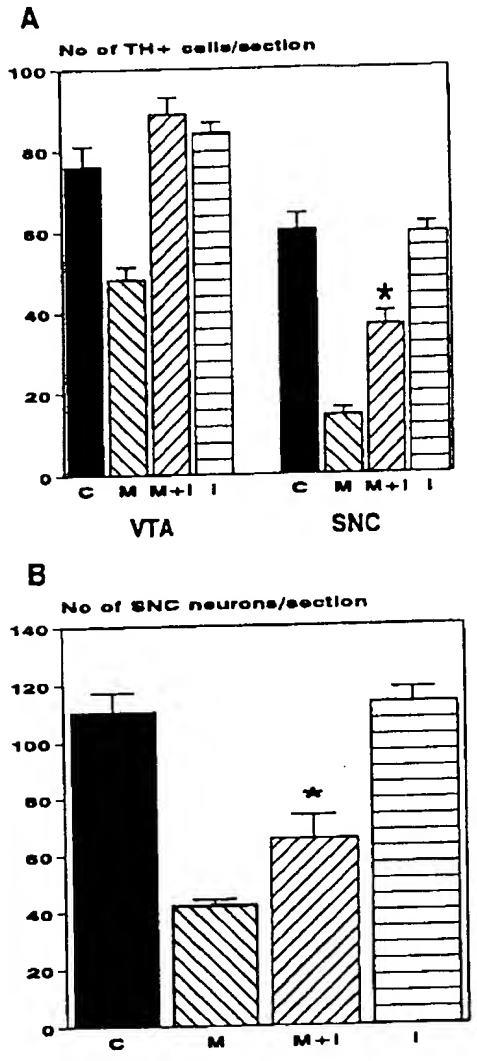


FIG. 4. Reduction of MPTP-induced loss of tyrosine hydroxylase (TH)-immunoreactive neurons in the substantia nigra compacta (SNC) and the ventral tegmental area (VTA) after injection of PDE IV inhibitors. Animals were given three injections of 20 mg/kg MPTP alternating with four isoquinoline injections at 1 h intervals, as outlined in Materials and methods. The brains were perfused and fixed 7 days later. (A) Cells stained for tyrosine hydroxylase immunoreactivity in the SNC and the VTA were counted in every second coronal 40 μm section between levels 46 and 54 in the atlas by Sidman *et al.* (1971), taken from five animals in each treatment group ($n = 60$). (B) Alternate sections were stained with toluidine blue and neurons in the SNC region were counted automatically using an MCID imaging system. With both staining methods used, the cell numbers in animals injected with MPTP plus inhibitor were significantly higher than in animals injected with MPTP alone ($^*P < 0.01$). C, vehicle control; M, MPTP-injected; M+I, injected with MPTP plus inhibitor; I, inhibitor alone.

cAMP does not reduce MPP⁺ uptake

The protective effect of phosphodiesterase inhibitors could occur at several steps in the cascade of MPTP action. If the uptake of MPP^+ into dopaminergic neurons was simply inhibited, our findings would be less interesting. Therefore, we investigated the possibility that the described effects of cAMP were due to reduced uptake of MPP^+ into dopaminergic neurons. Increasing cAMP levels in mesencephalic cultures had the same effect on [^{3}H]MPP^+ uptake as on dopamine uptake. When cultures were treated for 3 days with 3 μM forskolin, both dopamine uptake and MPP^+ uptake were increased to >150% of untreated control levels (Fig. 6B). Older cultures incubated on day 5 after plating with 500 μM cpt-cAMP showed equivalent levels of MPP^+ and dopamine uptake in treated versus untreated cultures (Fig. 6A). cAMP certainly protects against MPP^+-induced degeneration at these time points where no decrease in MPP^+ uptake can be observed (Hartikka *et al.*, 1992). We concluded that MPP^+ uptake was not decreased by elevated cAMP levels.

Discussion

We have shown that treatment of cultured dopaminergic neurons with PDE IV inhibitors, compounds which are known to increase cAMP levels, results in enhanced dopamine uptake and improved survival of the neurons and protects them against the specific dopaminergic toxin MPP^+. Treatment of C57BL/6 mice with these inhibitors substantially reduced the MPTP-induced dopamine depletion in the striatum and the decline in the number of SNC neurons as determined by tryosine hydroxylase immunocytochemistry and Nissl staining. It should be emphasized that the treatment with PDE inhibitors took place at the same time as the MPTP injections, beginning only 1 h before the first injection of MPTP and ending 1 h after the last. Similar results were obtained when the first MPTP injection was given 2 h after the last inhibitor injection, or the last MPTP injection was given 2 h before the first inhibitor injection (data not shown). A follow-up treatment was not needed for the observed reduction in the disappearance of dopaminergic neurons in the substantia nigra and in striatal dopamine depletion. This distinguishes our experiments from previous reports, where beneficial effects had been observed *in vivo* after long-term treatment with bFGF (Otto and Unsicker, 1990; Chadi *et al.*, 1993; Date *et al.*, 1993), ciliary neurotrophic factor (Hagg and Varon, 1993), epidermal growth factor (Hadjiconstantinou *et al.*, 1991), G_{M1} gangliosides (Schneider *et al.*, 1992; Herrero *et al.*, 1993) and glial-derived neurotrophic factor (GDNF) (Beck *et al.*, 1995). Previously, we found that bFGF enhanced dopamine uptake but it did not improve the survival of dopaminergic neurons *in vitro* nor did it protect them from MPP^+ toxicity under culture conditions where cAMP improved survival and resistance to MPP^+ (Hartikka *et al.*, 1992). Consistent with this, we propose that bFGF acts primarily by enhancing the recovery of the damaged neurons while protein kinase A phosphorylation actually protects the neurons from MPTP toxicity. This may be considered speculative since other authors described protective action of bFGF on dopaminergic neurons *in vitro*. This was, however, dependent on the culture conditions and the presence of astrocytes (Mayer *et al.*, 1993; Otto and Unsicker, 1993). Herrero *et al.* (1993) came to the conclusion that GM1 gangliosides exert neurotrophic action on the surviving neurons rather than protective effects. Ciliary neurotrophic factor was reported to transiently enhance tyrosine hydroxylase gene expression, leading to an apparent increase in the number of tyrosine hydroxylase-immunopositive cells rather than being actually protective (Magal *et al.*, 1993). It is particularly interesting that brain-derived neurotrophic factor (BDNF), initially characterized as a survival factor for dopaminergic neurons *in vitro* (Hyman *et al.*, 1994), also enhances function rather than survival *in vivo* (Knusel *et al.*, 1992; Sauer *et al.*, 1993). While members of the transforming growth factor protein family like TGFβ2 and TGFβ3 have been reported to increase the survival of dopaminergic neurons in culture, their effects *in vivo* remain to be shown (Lin *et al.*, 1993; Poulsen *et al.*, 1994). More recently, relatively small but significant protective and regenerative

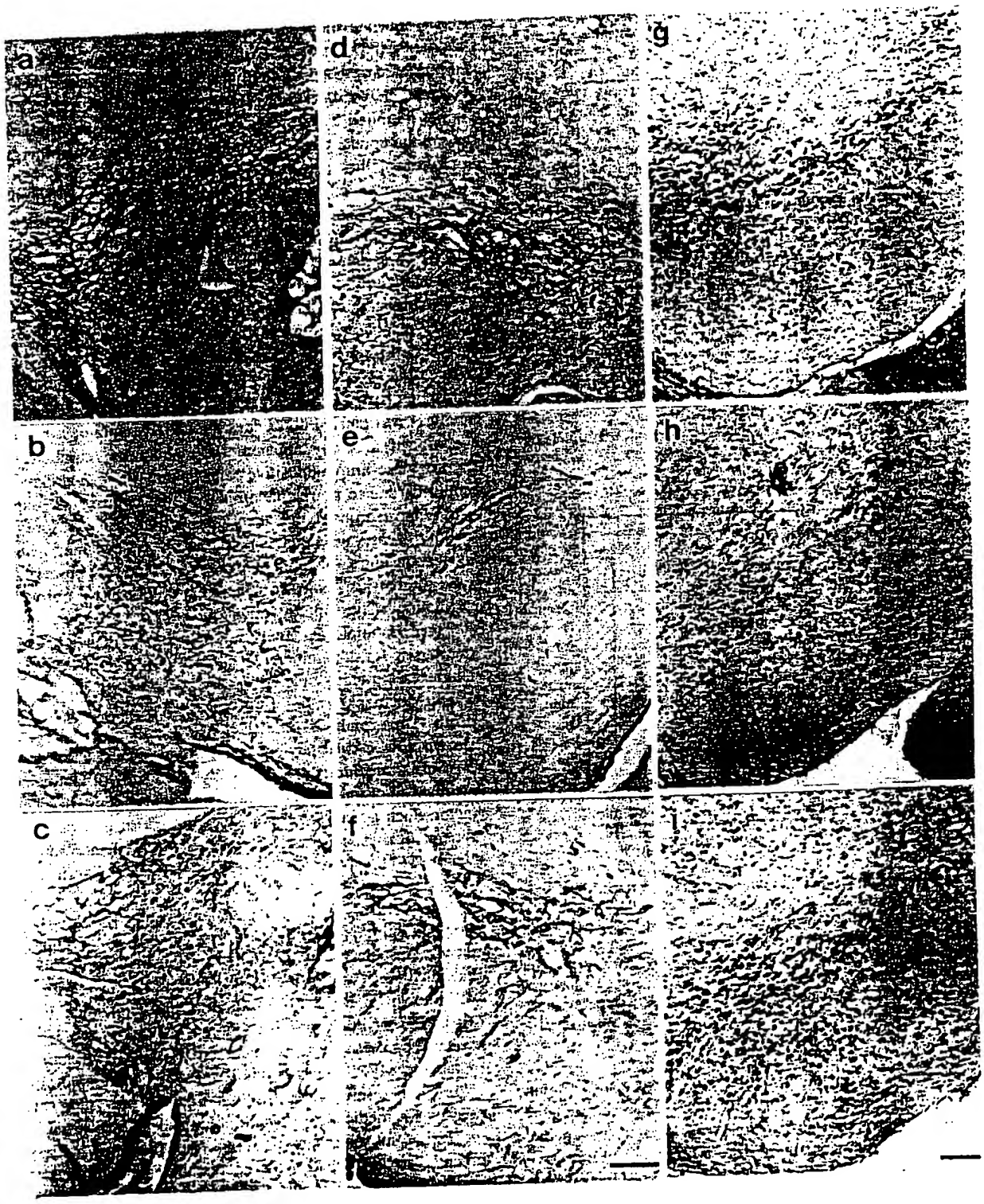


FIG. 5. Neurotoxic effect of MPTP on tyrosine hydroxylase-positive neurons in the ventral tegmental area (VTA) (a, b, c) and the substantia nigra compacta (SNC) (d, e, f, g, h, i) is abated by coinjection with the type IV PDE inhibitor. Representative brain slices from the same coronal level (slices in a, c, d and f are from the left, those in b, e, g, h and i from the right side of the brain) compared in control mouse brains (a, d, g), MPTP-treated animals (b, e, h) and animals injected with MPTP and the type IV PDE inhibitor (c, f, i). In mice injected with MPTP the number of tyrosine hydroxylase-positive neurons in the SNC (e) and to a lesser extent in the VTA (b), as well as the number of large neurons in the SNC (h), was severely reduced. Simultaneous injections with the isoquinoline showed a profound protective effect that was observed with both staining methods. PDE IV inhibitors alone did not cause any changes (data not shown). Injections were performed as described in the legend to Figure 4. Scale bars = 100 μm.

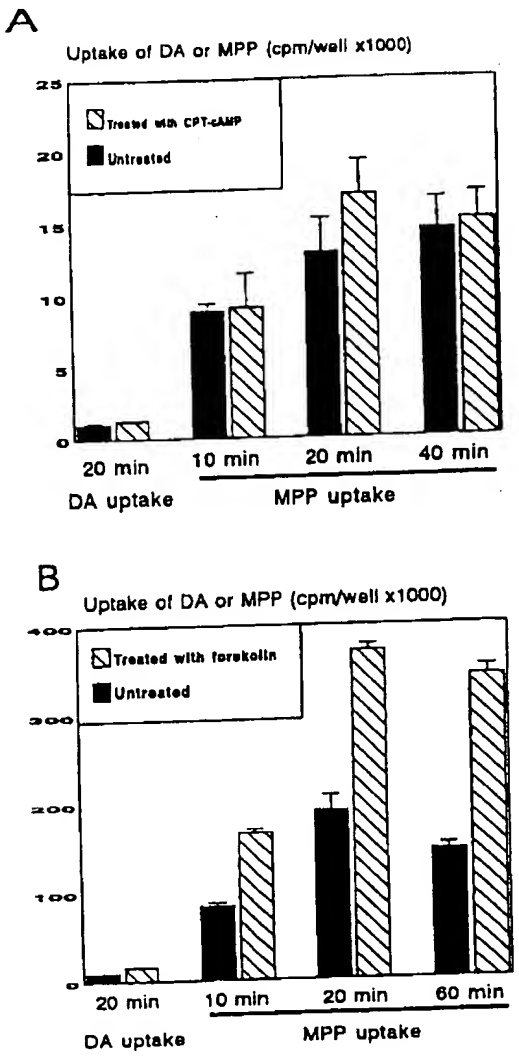


FIG. 6. Uptake of [^{3}H]MPP in midbrain cultures is not decreased by cAMP pretreatment. (A) Five-day cultures pretreated for 1 day with 500 μM CPT-cAMP (hatched bars) showed equivalent dopamine and MPP^+ uptake levels compared with untreated controls (solid bars) independent of the indicated uptake times. (B) Cultures treated with 3 μM of forskolin (hatched bars) for the first 3 days after plating showed a dopamine and MPP^+ uptake that was almost doubled compared to untreated controls (solid bars). Values are given ± SE, $n = 4$.

effects have been shown for GDNF in the MPTP-lesioned striatum and substantia nigra (Tomac *et al.*, 1995). GDNF also reduced the loss of tyrosine hydroxylase-positive neurons caused by medial forebrain bundle transection when administered by daily brain microinjections for 14 consecutive days prior to the lesion (Beck *et al.*, 1995). Controversial results were obtained with NMDA antagonists (Turski *et al.*, 1991; Zuddas *et al.*, 1992; Sonsalla *et al.*, 1992). The vigilance-promoting drug modafinil has also been shown to protect dopaminergic neurons from degeneration after MPTP injection (Fuxe *et al.*, 1992) and after partial transection of the dopamine pathway (Ueki *et al.*, 1993). While modafinil has an unknown mode of action, we describe here a general mechanism through which dopaminergic neurons can be protected from degeneration. It is an obvious thought that modafinil and other beneficial pharmaceuticals may in some way act through an enhancement of phosphorylation by protein kinase A.

The mechanism described is most interesting if it acts on the late steps of MPTP toxicity. Early steps may be model-specific and less important for the pathology of Parkinson's disease. We found that monoamine oxidase B (MAO-B), which oxidizes MPTP to MPP^+, is not inhibited by the PDE IV inhibitors (data not shown). Furthermore, the uptake of MPP^+ into mesencephalic dopaminergic neurons in culture is not reduced by elevated cAMP. Nevertheless, the PDE inhibitors protect dopaminergic neurons in culture from the action of MPP^+. Therefore, we assume that MPP^+ does in fact enter the dopaminergic neurons but its toxic action is interrupted by a protein kinase A-dependent mechanism at a point that requires further characterization.

Tyrosine hydroxylase gene expression is under the control of a cAMP-responsive element (Lewis *et al.*, 1987). After local injection of forskolin into the substantia nigra, tryosine hydroxylase gene expression was found to be up-regulated for 1–2 days with no apparent change in protein levels (Leviel *et al.*, 1991). Although our analysis was carried out 7 days after PDE inhibitor injection, it may be argued that any apparent increase in the number of tyrosine hydroxylase-immunopositive neurons under our treatment conditions could be due to up-regulation of tyrosine hydroxylase expression in surviving neurons, rather than a protective effect. To prove that this is not the case, we also counted toluidine blue-stained neurons in the substantia nigra. A similar protective effect was observed with this type of analysis, indicating that we are not simply observing an up-regulation of tyrosine hydroxylase.

There is ample evidence in the literature that MPTP is strongly neurotoxic for dopaminergic neurons and that treated neurons display characteristic signs of swelling and neurite distortion (Burns *et al.*, 1983; Mochizuki *et al.*, 1994), possibly followed by apoptotic cell death (Dipasquale *et al.*, 1991; Mochizuki *et al.*, 1994). It is not known how cAMP prevents these toxic events. Currently, it is not possible to decide if it acts directly via a growth factor-independent mechanism or indirectly by influencing the response to a neurotrophic factor. Such a situation has been described for glutamate, which acts synergistically with nerve growth factor (via metabotropic glutamate receptors) to improve the survival of cerebellar Purkinje cells but is inactive alone (Mount *et al.*, 1993).

Regardless of the mechanism, our results suggest that type IV-specific inhibitors or other cAMP-modulating compounds may act to retard neuron death in Parkinson's disease. We have no direct evidence that all of our substances cross the blood–brain barrier, but this is well known for Ro-201724 (Mumford and Holtzman, 1990) and for other PDE IV inhibitors like rolipram (Muller *et al.*, 1993). We see the same protective effects against MPTP *in vivo* as against its active metabolite MPP^+ *in vitro*, where there is no blood–brain barrier to cross. It therefore seems that our findings may represent a viable concept for medical treatment, unhindered by difficulties in applying the active substances to the brain. Nevertheless, other points like potential toxicity need to be addressed. Toxicity did, however, not pose a major problem when PDE type IV inhibitors were clinically tested for the treatment of depression (Muller *et al.*, 1993). Molecular cloning has revealed the existence of at least four different PDE IV subtypes, which provides an additional potential for specificity (Michaeli *et al.*, 1993; Engels *et al.*, 1995). The beneficial effect of the inhibitors may be synergistically enhanced (compare Fig. 1A and B) by the combined application of an agonist for a receptor which stimulates adenylate cyclase in the relevant cells. Such a combination could in addition improve the specificity of the treatment since cAMP levels would be increased predominantly in cells which express both the receptor and the particular PDE IV subtype. It will

be important to identify the proteins which improve the survival of dopaminergic neurons after protein kinase A phosphorylation and to analyse the relationship between these proteins and the biochemical events which underlie the degeneration of dopaminergic neurons in Parkinson's disease.

Acknowledgements

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Abbreviations

bFGF	basic fibroblast growth factor
CPT-cAMP	8-(4-chlorophenylthio)-cAMP
EDTA	ethylenediamine tetraacetic acid
GDNF	glial-derived neurotrophic factor
MPP^+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NMDA	*N*-methyl-D-aspartic acid
PDE	phosphodiesterase
SNC	substantia nigra compacta
VTA	ventral tegmental area

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